An-Najah National University

Faculty of Graduate Studies

Plasma MicroRNA Biomarkers for Early Detection of Lung Cancer among People Exposed to Diesel Exhaust Emissions in Palestine

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Dedication

This thesis is dedicated to:

The pupils of my eyes who have always been a source of inspiration, and stamina, my grateful parents

To the flower of my heart, my lovely wife, for supporting and encouraging me all the time, and to her grateful family

To my lovely daughters and my happiness Hanan and Bisan who are indeed a treasure from Allah

To my great brothers and affectionate sisters and my all friends

To all cancer patients having a hope for cure

To Palestine, my homeland

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Ahmad Isam Mohammad Slaileh

الإقرار

أنا الموقع أدناه مقدم الرسالة التي تحمل العنوان:

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أقر بأن ما اشتملت عليه هذه الرسالة هي نتاج جهدي الخاص، بإستثناء ما تمت الإشارة إليه حيثما ورد، وأن هذه الرسالة كاملة، وأي جزء منها لم يقدم من قبل لنيل أي درجة أو لقب علمي أو بحثي لمؤسسة تعليمية أو بحثية أخرى.

Declaration

The work provided in this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification.

Student's Name: **اسم الطالب:**

Signature: **التوقيع:**

Date: **التاريخ:**

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List of Abbreviations

PHIC	Palestinian Health Information center
EGFR	Epidermal Growth Factor Receptor
RB	Retinoblastoma Tumor Suppressor Gene
HNPCC	Hereditary nonpolyposis colorectal cancer
SCLC	Small cell lung carcinoma
NETs	Neuroendocrine tumors
WHO	World Health Organization
IASLC	International Association for the Study of Lung Cancer
NSCLC	Non-small cell lung carcinoma
SCC	Squamous cell carcinoma
LCC	Large cell carcinoma
PAHs	Polycyclic aromatic hydrocarbons
DME	Diesel motor emissions
IARC	International Agency for Research on Cancer
RR	Relative risk
CI	Confidence interval
OR	Odds ratio
CT	Computed tomography
PET	Positron emission tomography
UICC	Union for International Cancer Control
TNM	Tumor-nodes-metastasis
GEFs	Guanine nucleotide exchange factor
Raf	Serine/threonine kinase
MEK	MAP kinase kinase
ERBB2	Erb-b2 receptor tyrosine kinase 2

IGF-I	Insulin-like growth factor I
UTR	Untranslated region
Pri-miRNA	Primary miRNA
Pre-miRNA	Precursor miRNA
DGCR8	DiGeorge syndrome critical region gene 8
RISC	RNA-induced silencing complex
Ago2	Argonaute 2
TRBP	Transactivation-responsive RNA binding protein
ac-pre-miRNA	Ago2-cleaved precursor miRNA
NPV	Negative predictive value
PPV	Positive predictive value
DNMTs	DNA methyltransferases
HDACs	Histone deacetylases
HMTs	Histone methyltransferases
DNMT3A	DNA methyltransferase 3 alpha
DNMT3B	DNA methyltransferase 3 beta genes
TPM1	Tropomyosin1
PTEN	Phosphatase and tensin homolg
PDCD4	Programmed cell death 4
TIMP2	Tissue inhibitor metallopeptidases 2
EDTA	Ethylenediaminetetraacetic acid
OD	Optical density
cDNA	Complimentary DNA
AGE	Agarose Gel Electrophoresis
IRB	Institutional Review Board
MOH	Ministry of Health
TBE	Tris Borate EDTA

PCR	Polymerase chain reaction
T_m	Melting temperature
MYC, Kras, HRAS, NRAS, BCL2, PK3CA	Proto-oncogenes
ERK1, ERK2	Mitogen-activated protein kinase 3
Ets, Fos	Transcription factors
TGFα	Transforming growth factor alpha
TP53, MDM2, CDKN1A, FAS, SEN, DDB2, BAX, GADD45A, MLH1, MSH2, DPC4, MLL2, PTEN	Tumor suppressor genes
BIRC5	Baculoviral IAP Repeat Containing 5 gene
p53	Tumor suppressor
ERK1, ERK2	Mitogen-activated protein kinase 3 genes
NTC	Non-template control

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Abstract

Background:

Lung cancer is the most common cancer among males in Palestine, and it is closely related to environmental factors such as diesel exhaust emissions which leading to genetic instability. MicroRNAs are a group of small non-coding RNAs which consist of 19 to 25 nucleotides and have many different roles at posttranscriptional level either by mRNA degradation or prevent the translation process. MiRNAs regulate about 30% of human genes involved in cellular proliferation, differentiation, and apoptosis. Moreover, miRNAs act as either tumor suppressors which prevent tumor formation or oncogenes which target tumor suppressor and apoptotic genes. Up-regulation of both miR-21 and miR-15b were observed in lung cancer patients and also in people exposed to diesel motor emissions.

Objectives:

This study screened miR-21 and miR-15b as non-invasive and cost effective plasma biomarkers test for early detection of lung cancer among

people work at transportation center in Nablus city and exposed to high level of DME.

Methods:

A total of 20 peripheral blood samples including lung cancer patients, healthy relatives, and high risk people were evaluated in this study. MiRNAs were extracted from plasma, and then poly adenylated using poly (A) polymerase, cDNA was synthesized using reverse transcriptase, and then quantity of miR-21 and miR-15b were calculated by absolute quantification real-time PCR (qPCR).

Results:

Over-expression of miR-21 and miR-15b was found in 90% and 100% of all high risk people exposed to DME respectively. All lung cancer patients were showed over-expression in both miR-21 and miR-15b. Forty percent of healthy control were showed an elevation in the expression of both miR-21 and miR-15b compared with Cel_miR-39 spike-in control. The sensitivity and specificity were 90% and 60% for miR-21, while 100% and 60% for miR-15b respectively. PPV and NPV were 81.8% and 75% for miR-21, while 83.3% and 100% for miR-15b. Significantly, both miR-21 and miR-15b were over-expressed in DME exposed people and lung cancer patients compared with healthy control. The results also show the correlation between the exposure to DME and the plasma miRNAs levels, the odds ratio was OR=28.5 (95% CI 2.6-306) for both miRNAs.

Conclusion:

MiR-21 and miR-15b could be a potential non-invasive and cost effective biomarker for early detection and diagnosis of lung cancer. Also, this transportation center is unfit for human working and may lead to lung cancer development for drivers and workers.

Chapter One

Introduction

1.1 Overview

Cancer is set of diseases with the common feature of unregulated cell growth and one of the major public health problems worldwide, which is threatening human life (1). The cancer death rate has increased in developing countries such as Asia, Africa, and Latin America; due to the increases of smoking, excess body weight, and their poor lifestyle. As a result, the predicted number of cancer incidence will be increasing by 2020 to be 21.7 million in contrast to 14.1 million cases in 2012 (2, 3).

Malignant neoplasm, a disease characterized by uncontrolled cellular proliferation leading to abnormal mass of cells or tumor, which invades (metastasize) the surrounding tissues and spreads far off the original site. Moreover, there are three main classes of cancer, sarcomas, carcinomas, and hematopoietic and lymphoid malignant neoplasm (4).

1.2 Epidemiology of Cancer in West Bank

Based on the data published by Palestinian Health Information center (PHIC) in 2018, 3102 new cancer cases were diagnosed in west bank, 1565 cases were reported among females (50.5% of all reported cases) and 1537 cases were in males (49.5% of all reported cases). The incidence rate of reported cancer cases (Figure 1.1) shows a noticeable rise in the last nine years (2010 to 2018), the incidence rate was increasing from 53.7 in 2010 to 117.7 per 100,000 populations in 2018 (5).

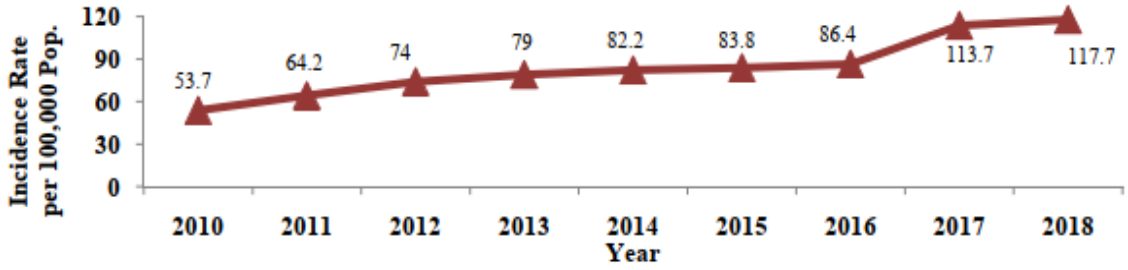


Figure 1.1 Reported cancer incidence rate per 100,000 population from 2010 to 2018, West Bank, Palestine 2018 (5).

According to the reported cancer cases by the Palestinian Ministry of Health in 2018, breast cancer was the most common with 439 cases, the incidence rate was 16.7 per 100,000 population (Figure 1.2) with 14.2% of all reported cancer cases (Figure 1.3). While 358 colorectal cancer cases reported to put it in the second most common cancer with incidence rate 13.6 per 100,000 population and 11.5% of all cancer cases. The third common reported cancer cases is lung cancer, 249 lung cancer cases reported with incidence rate 9.4 per 100,000 population and 8% of all cancer cases (5).

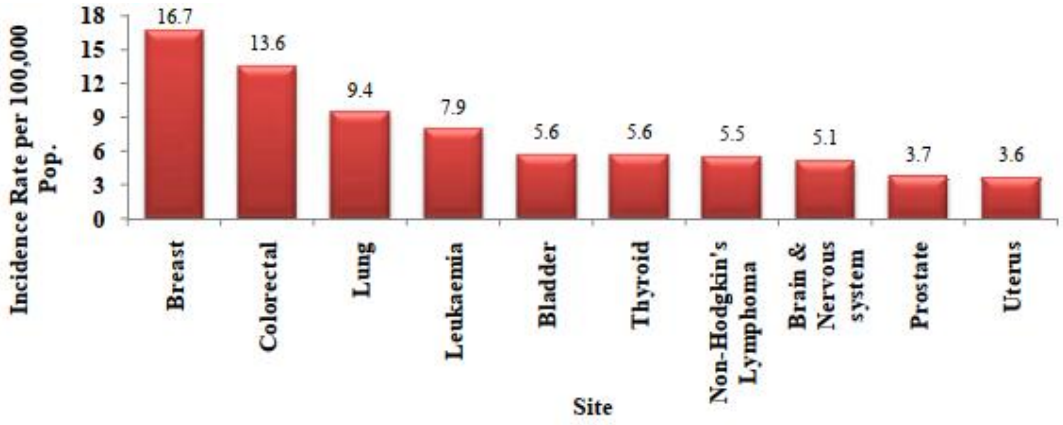


Figure 1.2 Incidence rate of most common cancer cases in West Bank in 2018 (5).

Figure 1.3 percentage of most common cancer cases in West Bank in 2018 (5).

1.3 Cancer Genetics

Many different genetic and epigenetic alterations can lead to cancer. A driver gene mutation, is mutation that causing development or progression of cancer (4). A normal cell evolves into cancer cell when multiple changes occur at the levels of some genes that have a role in regulation of cellular proliferation. These cancer cells can invade tissues, evade of apoptosis, sustain angiogenesis (formation of blood vessels), and replicate unlimitedly (6). However, there are two major classes of normal regulatory cellular genes in the human genome that have ability to cancer cell development, which are:

1) Proto-oncogenes, are normal genes encode for proteins that have main roles in regulation of normal cellular growth, including differentiation, proliferation, and survival. For example, some proto-oncogenes code for proteins act as growth factors in cellular communication pathway. Also, some of these proteins are cell surface receptors such as EGFR (Epidermal Growth Factor Receptor). Others, such as myc, are transcription factors which manipulate gene expression by binding directly to DNA molecule.

Another proteins such as ras act as plasma membrane GTP-binding protein (6). However, viral infection, point mutation, genetic amplification, and chromosomal translocation may convert proto-oncogenes into oncogenes by changing either gene structure or regulatory region, which lead to overexpression of these proteins, and responsible for cancer cell development (Figure 1.4). About 30 percent of human cancers have an active oncogenes which could be sufficient to promote cancerous cells. Usually, only one allele required to be mutated to cause cancer. Focusing on lung cancer, *D*1, C-*myc*, L-*myc*, N-*myc* by genetic amplification, K*ras* by point mutation are oncogenes that may be unregulated and lead to lung cancer (7).

2) Tumor suppressor genes, encode for proteins that have four functional roles. First, suppress the abnormal progression of cells during cell cycle such as retinoblastoma (*RB1*) gene. Second, inhibit metastasis and prevent spreading of cancer cell by proteins involved in cell adhesion. Third, DNA repair proteins such as those encoded by *MSH2* and *MLH1* genes. Finally, there are proteins that trigger apoptosis when the repair of the DNA damage is too severe (8, 9). However, tumor suppressor genes follow the two-hit hypothesis or known as Knudson hypothesis, which means that both gene copies must be mutated to generate an abnormal cell which lead to loss of their functions in cancer suppression. Tumor progress when germline mutation occurs in one allele of a tumor suppressor gene and somatic mutation of another allele occur during the individual lifetime (10). Also, there are two categories of tumor suppressor genes, the first one called gatekeepers genes which directly suppress growth and proliferation of

abnormal cells such as *Tp53* and *Dpc4* genes. Second called caretaker genes which prevent genetic instability and essential for DNA repair (11). Loss of caretaker genes function by a mutation of DNA repair genes such as Hereditary nonpolyposis colorectal cancer (HNPCC) genes *MLH1* and *MSH2* will increase the rate of DNA mutation, thereby mutation of gatekeepers genes will increases (12).

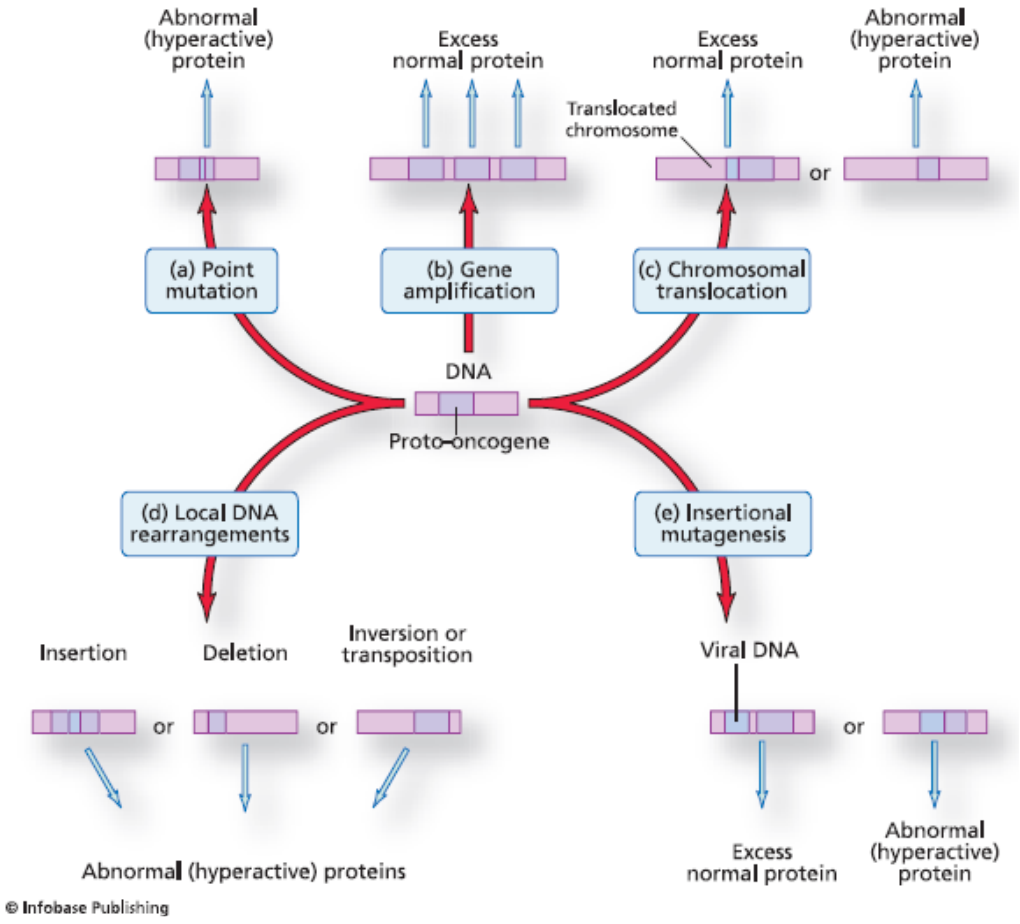


Figure 1.4 Methods clarify transforming proto-oncogenes into oncogenes (6).

1.4 Lung cancer

1.4.1 Lung anatomy and physiology

Lung is sponge-like organ, composed of three lobes in the right lung and two lobes in the left one. During breathing, air moves toward the lung through trachea, which divides into right and left tubes called bronchi. Each bronchus branches into smaller tubes called bronchioles, which end with alveoli (Figure 1.5) (13).

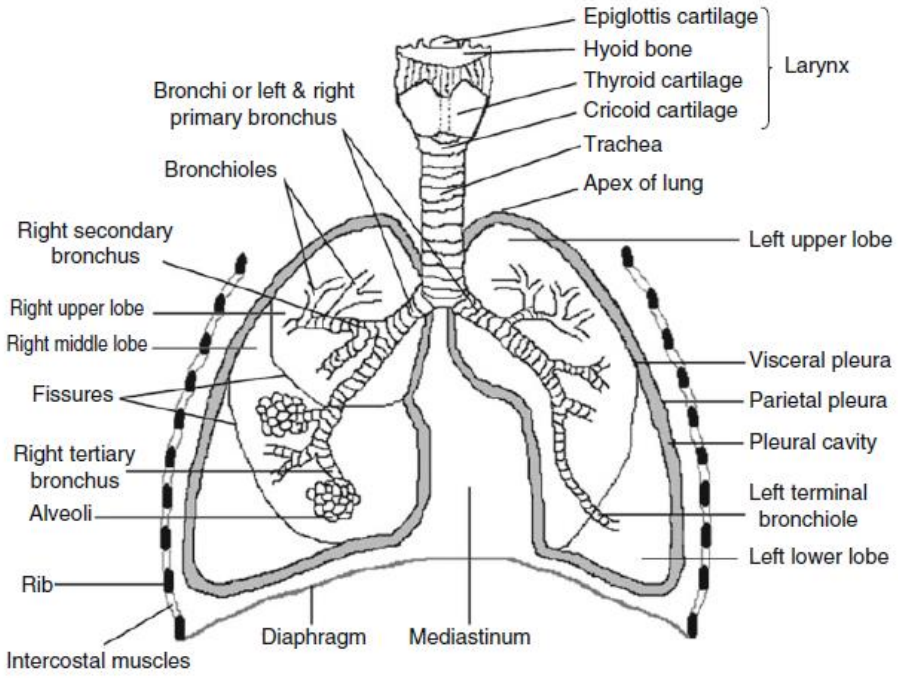


Figure 1.5 The general anatomy of the lung (14).

1.4.2 Overview of lung cancer

Lung cancer is a global problem, and the most common type of cancer causing death (15). In addition, it was responsible for 1.37 million deaths in 2017 in Europe (16). The prevention and treatment of lung cancer are successfully achieved when it was early detected (17). Moreover, 60-80%

survival at 5 years is investigated in patients diagnosed with early stage disease. Although the early diagnosis of lung cancer is possible through tomography-based population screening, it needs a specialized health center, and there is a difficulty to detect individuals at high risk without symptoms of lung cancer (18).

1.4.3 Histopathology of lung cancer

There are two major clinic-pathological groups of lung cancer:

a) Small cell lung carcinoma (SCLC), about 25% of all lung cancer cases in Europe as well as in USA (19). The name is come from the small size and appearance of the cancer cells and almost caused by smoking. This type of lung cancer begins in bronchial location and lead to obstruction of bronchi. SCLC also classed as neuroendocrine tumors (NETs), which begins in the neuroendocrine cells of the lung. These cells are responsible for hormones secretion that regulate the blood and air flow in the lungs (15).

Small cell lung carcinoma subtypes including: (1999 WHO/IASLC classification).

1- Small cell lung cancer (with pure histology).

2- Combined small cell lung cancer (combined with non-small cell cancer type).

b) Non-small cell lung carcinoma (NSCLC), which has three major histological types:

1) Squamous cell carcinoma (SCC), which develops from the flat cells that line the surface of respiratory tract, also it is strongly correlated with smoking. Many tumor suppressor genes mutations such as *TP53*, *MLL2*, and *PTEN*, and oncogenes mutations such as *PK3CA*, and *EGFR* were observed in SCC cases (15, 20).

2) Adenocarcinoma, the most common type of lung cancer. It begins in mucus producing-glandular cells of the lung, and it tends to spread out of the body in early stage. Moreover, the frequent histological heterogeneity make adenocarcinoma more complicated than other lung cancer types, since mixture of adenocarcinoma subtypes are more common (15).

3) Large cell carcinoma (LCC), undifferentiated tumor that lacks histological features of squamous or glandular differentiation. Histologically, large cell carcinoma is composed of nest large polygonal cells with vesicular nuclei and prominent nucleoli (13, 15, 21, 22).

1.4.4 Epidemiology of lung cancer

According to the World Health Organization (WHO), worldwide lung cancer is the most common type of cancer was diagnosed with 2.09 million cases, and the most common causes of cancer death with 1.76 million deaths in 2018 (23). On another hand, lung cancer is the most common cancer among males population in Palestine (Figure 1.4). There are 207 lung cancer cases reported in 2018, the incidence rate was 15.4 per 100,000 population, representing 13.5% of all cancer cases among males. Also, lung cancer is the

first leading cause of cancer death with 19.6% of those who died by cancer in Palestinian population (5).

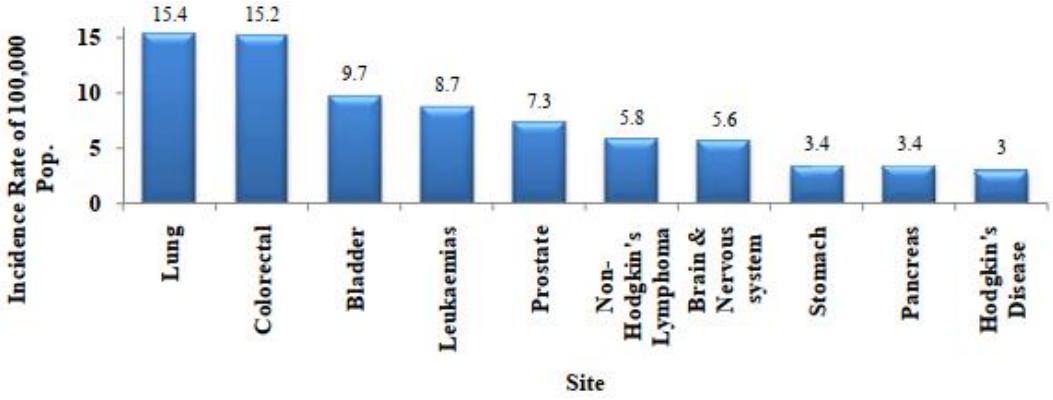


Figure 1.6 Incidence rate of most common cancer cases among males in West Bank at 2018 (5).

1.4.5 Etiology of lung cancer

Lung cancer is the major reason for cancer death in 21st century. It is closely related to physical and environmental factors which leading to genetic instability and tumor development. These factors include smoking, air pollutions, radon, asbestos, workplace factors, and viruses (15). Although about 85% of lung cancer patients are attributable to cigarette smoking, lung cancer develops only in about 15% of smokers (24). However, it is relatively affects men more than women. Generally, exposure to smoking in any form increases the risk of lung cancer, including secondhand smoking, hookah, and cigar smoking (25). There are approximately 70 carcinogens out of 4000 compounds found in cigarette, these carcinogenic compounds such as polycyclic aromatic hydrocarbons (PAHs) are responsible for DNA adducts in *TP53* gene, which generate lung tumor (15).

Moreover, cancer is a disease which strongly linked to the human lifestyle. Cancer causing agents or carcinogens, which may be found in food, water, air, and chemicals are responsible for developing malignant tumors, one of these chemicals is diesel exhaust gas or diesel motor emission (DME) (1, 2).

1.4.6 Diesel exhaust gases and lung cancer

Many intensive researches are focusing on the health effects of the diesel exhaust, which contains mutagenic and carcinogenic particles, especially nitrous oxides, polycyclic aromatic hydrocarbons (PAH) and nitroarenes (26, 27, 28, 29). Acute exposure to these small particles of diesel exhaust is strongly associated with changes of lung function, respiratory symptoms, and even neurophysiological symptoms (30, 31).

Diesel motor emissions (DME) are a complex mixture of particles and gases containing hundreds of chemical compounds. Diesel exhaust particles are composed of a center core of elemental carbon and adsorbed organic compound, in addition to few amounts of nitrate, sulfate, and metals. While nitrogen compounds, carbon monoxide, sulfur compounds, and many hydrocarbons compounds such as benzene, aldehydes, polycyclic aromatic hydrocarbons (PAHs), and nitro-PAHs are classified as gaseous components (Figure 1.7) (31). Since these particles have large surface area and small size, they can reach the deep lung and even the blood stream and lead to lung cancer (32).

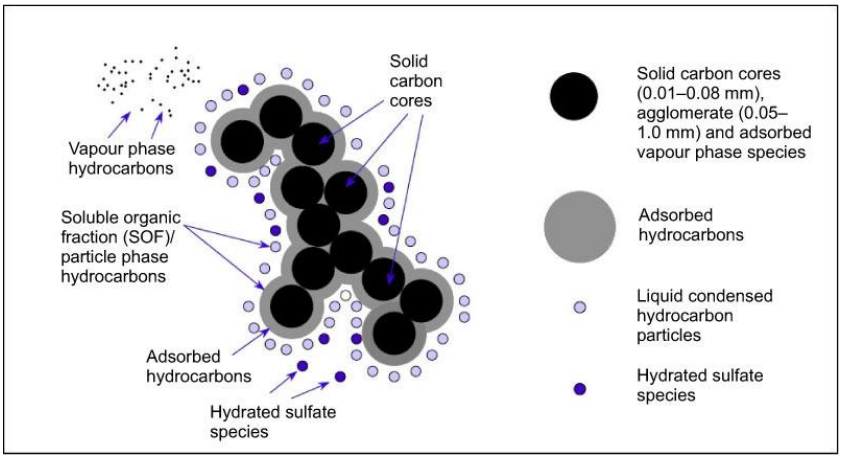


Figure 1.7 Diesel exhaust particles composition (33).

The International Agency for Research on Cancer in 1989 [IARC, 1989] concluded that diesel exhaust is a carcinogenic substance depending on lung cancer detected in experimental animals (34). Furthermore, the emissions of diesel exhaust not only contain highly mutagenic substances, but also increase levels of DNA adducts in peripheral blood cells. (35, 36, 37).

In 1990, cancer incidence was investigated among 695 bus garage workers including a mechanic, and serviceman exposed to diesel exhaust for at least six months in Stockholm. The relative risk (RR) for lung cancer was 2.4 (95% confidence interval (CI) 1.3 – 4.5) among men who exposed to emissions of diesel exhaust at high rate compared with low rate of exposure. As a result, the study indicated that lung cancer risk increased by increasing the exposure intensity of diesel exhaust gases (38).

Lung cancer mortality was assessed between 1959 and 1996 for 54973 U.S. railroad workers. This study was showed elevated lung cancer risk among engineers, conductors, firemen, and brakemen, since these entire job

categories exposed to high intensity of diesel exhaust emissions. Moreover, 4351 case of lung cancer deaths were also observed with a relative risk of lung cancer mortality of 1.40 (95% CI 1.30–1.51) (39).

A study in Germany on drivers of taxies, buses, lorries, forklift trucks, tractors, bulldozers, and diesel locomotives, were certainly exposed to diesel motor emission (DME) were evaluated of lung cancer risk. The study provides an evidence that occupational exposure to DME is correlate with high risk of lung cancer depends on the results that showed an odds ratio of OR= 1.43 (95% CI 1.23-1.67) for all jobs (40).

1.4.7 Clinical manifestation of lung cancer

The incidence and mortality rates of lung cancer are expected to increase in all countries as a result of tobacco smoking and other carcinogenic factors such as DME (41). Although there are no signs or symptoms in the early stages of tumor development, about 90% of lung cancer cases show symptoms at the diagnosis time. Primary tumor, intrathoracic or extrathoracic spread responsible for direct signs and symptoms which reveal on patients (42). However, there are some differences in secondary signs and symptoms depending on the tumor region. Cough, dyspnea, hemoptysis, wheeze and stridor, and postobstructive pnuemonitis appear in the central growth of tumor. While symptoms for the peripheral growth of tumor including pain (from pleural wall), cough, dyspnea, and lung abscess as a result to tumor cavitation (presence an air space within tumor) (43).

Approximately 10% of lung cancer patients develop paraneoplastic syndrome which caused by bioactive substances formed by cancerous cells. These syndromes include hypercalcemia, Cushing's syndrome, hypertrophic pulmonary osteoarthropathy, Lambert-Eaton myasthenic syndrome, and cortical cerebellar degeneration (44).

1.4.8 Diagnosis and staging

The evaluation and accurate diagnosis of tumor tissue should be established for management of lung cancer. Treatment and prognosis estimation of the lung tumor is highly depends on the histological cell type, and the accurate staging of malignancy (13). Although five-year survival for stage I reach 70%, less than 20% of lung cancers are early detected. The vast majority of early stage detected lung cancer is asymptomatic and investigated incidentally using chest radiograph (chest X-ray) or computed tomography scan (CT scan) achieved for other reasons. However, chest radiograph or CT scan are the first tests performed when signs and symptoms reported on patient. Moreover, tumor histopathology can be obtained by bronchoscopy or CT-guided biopsy (45).

Sputum cytology, flexible bronchoscopy, and transthoracic needle aspiration are techniques available when the lung cancer type and stage are not clear (42).

The molecular analysis for early detection of lung cancer among asymptomatic persons was proved its efficiency, since sputum cytology and chest radiographs screening procedure showing some disadvantages.

Although these approaches improve the diagnosis of lung cancer, no improvement of lung cancer mortality was observed. Ultimately, 70% of lung cancer patients diagnosed with stage III or VI. Early detection of lung cancer using plasma or serum biomarkers has preference since it is simple, noninvasive, cost effective, and reliable diagnostic test (13).

The appropriate treatment and prognosis evaluation of lung cancer patient are achieved by a precise diagnosis and staging of lung cancer. Staging is the assessment process that can detect the spreading of cancerous cells from the original site (46). Positron emission tomography (PET) scan enhance the staging, and it is carried out when metastatic lung cancer is suspected (47). The classification of staging proposed by the International Union against Cancer (UICC) depends on the type of tumor and the evaluation of metastasis. Stages of NSCLC classified from one to four (I, II, III, VI). The stages depend on spreading of tumor, more spreading of cancer considered a high stage (48). Evaluation of NSCLC based on international TNM staging system (tumor-nodes-metastasis). This classification clarifies the range of primary tumor, the involvement of lymph node, and presence or absence of metastasis. Whereas small cell lung cancer classified as either limited stage which means the tumor confined to ipsilateral hemithorax (found in one side of chest), or extensive stage which means outside the ipsilateral hemithorax (spread to other parts) (42).

1.4.9 Treatment of lung cancer

Lung cancer treatment actually depends on many factors including tumor cell type, metastasis distant, and performance status of patient. Surgery is the primary treatment for resectable patients diagnosed with early stage of NSCLC (stage I and II). It provides 60%-80% of five-year survival rates for stage I NSCLC and 30%-50% for stage II patients. Whereas radiotherapy such as stereotactic body radiotherapy (SBRT) could be applied for an unresectable tumor or high-risk cancer patients (49, 50).

The management strategy used for stage III NSCLC patients including chemotherapy, radiotherapy, and surgery, which depends on tumor location and surgical resection possibility. However, stage III NSCLC is a cellular and genetic heterogeneous disease. Heterogeneity of stage III NSCLC refers to tumor location, tumor genomics, and histological subtypes (20, 51). Many clinical studies showed a chance for improvement of five-year survival rate about 6% by using neoadjuvant chemotherapy (52).

Many factors such as performance status (PS) of patient, comorbidity, and histological and genetic features of tumor determine the treatment strategy of stage IV NSCLC. Forty percent of NSCLC patients are diagnosed in stage IV (53). The primary treatment of this stage including palliative radiotherapy (reduce tumor-related symptoms, such as bleeding and pain, which increase survival time), combination chemotherapy (more than one drug), combination chemotherapy with targeted therapy, epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor which block EGFR such as

Gefitinib and Erlotinib. While a combination of chemotherapy and radiotherapy are the best treatment option and lead to more response in case with SCLC (54).

1.5 Lung cancer genetics

In cancerous cells, an irregular growth signaling occurred due to activation of oncogenes. There are two ways for aberrant signaling:

1- Mimicking the other growth factors which promote cell growth

2- Canceling out other growth inhibitors

Mutations in human lung tumor found in many different genes including tumor suppressor genes, oncogenes, telomerase genes, cell death regulatory genes, and genes responsible for DNA repair. These mutations range from missense mutation to gene deletion and amplification (55).

1.5.1 Oncogenes

Ras superfamily GTPases proteins encoded by three different human *RAS* genes (*KRAS*, *HRAS*, and *NRAS*) and placed at the inner surface of plasma membrane. Moreover, Ras proteins have an initial role in cellular proliferation (56). Activation of Ras protein in form of GTP-bound occurred by guanine nucleotide exchange factor (GEFs). Activated Ras interacts with Raf (serine/threonine kinase) that activate MAP kinase kinase (MEK), leading to activation of mitogen-activated protein kinases ERK1 and ERK2. These proteins enter the nucleus and phosphorylate Ets, myc, and Fos

transcription factors which in turn promote cyclin D1 and many other growth factors transcription (57).

In lung cancer, studies reported an oncogenic activation of *KRAS* in 20-30% of NSCLC tumors (58). The over-expression mutation of *KRAS* occur significantly in adenocarcinoma when G to T transversion in codon 12, which induces lung tumor formation and motility of adenocarcinoma cells (59, 60, 61).

In 2000, a study was shown that Epidermal growth factor receptor gene which code for EGFR, and erb-b2 receptor tyrosine kinase 2 gene (*ERBB2)* which code for neuregulin receptor are both over-expressed by amplification mutation in NSCLC patients (62). In SCLC, insulin-like growth factor I (IGF-I) and its receptor are noticed to be over-expressed also. However, EGFR is a transmembrane protein and has a role in regulation of epithelial cell proliferation and inhibition of apoptosis as a response of EGF and TGFα ligands (15).

1.5.2 Tumor suppressor genes

TP53 gene is the most coding gene exposed to mutations in a broad different cancer types. It contains 11 exons and located at 17p13.1 (63). *TP53* gene codes for a nuclear protein called p53, which implement many functions in the cell, summarized in:

(1) p53 is a transcription factor that stimulate the expression of multiple genes by binding to DNA once activated, these genes including *MDM2*,

CDKN1A, *FAS*, *SEN*, *DDB2*, *BAX*, and *GADD45A*, which mediates regulation of cell cycle checkpoints, DNA replication, apoptosis, and repairing of DNA damage (64, 65).

(2) P53 has a key role in controlling apoptosis in response to oncogene expression and DNA damage by up-regulating *BAX* and *FAS* pro-apoptotic genes, and down-regulating *BCL2* and *BIRC5* (survivin) genes which inhibit apoptosis (57).

(3) P53 plays an effective role in repairing of DNA mutations through p53 regulated genes such as GADD45, and DDB2 (66).

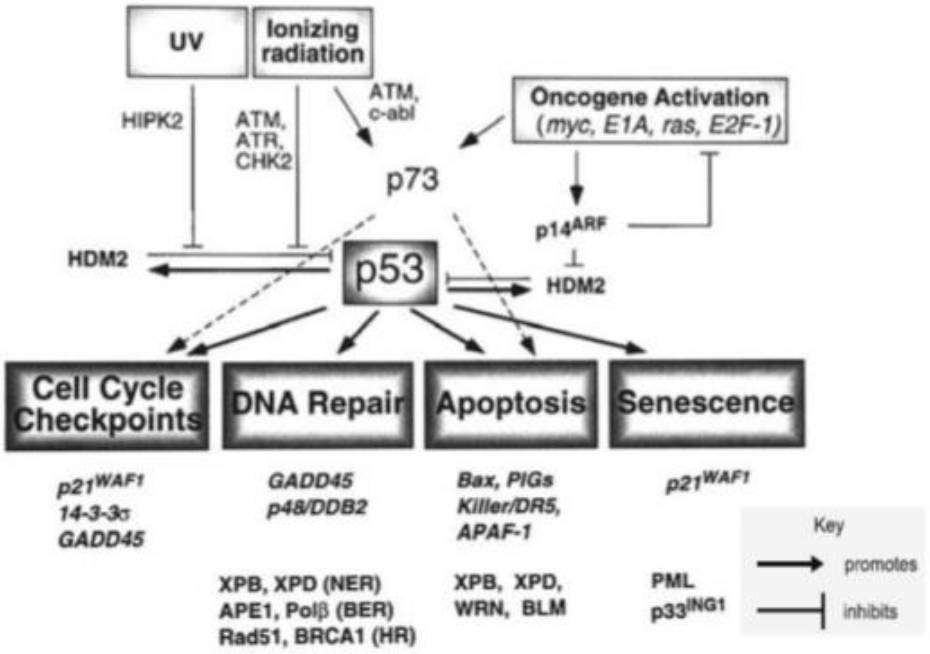


Figure 1.8 Effects of p53 activation in response to stress (67). NER: nucleotide excision repair. BER: base excision repair. HR: homologous recombination.

Molecular changes including, oncogenes and tumor suppressor genes mutations, extensive chromosomal rearrangements, and deregulation of different miRNAs were observed in tumor tissues and cell lines of lung

cancer. On another hand, susceptibility to lung cancer may be affected by many factors including differences in carcinogen metabolism and detoxification, cell cycle control, DNA repair, and programmed cell death (apoptosis) (1, 68, 69).

Loosing of p53 functions leading to increases of mutation rates not only at the genomic level but also at the level of chromosomes (70). Genetic abnormalities including defects in tumor suppressor genes, such as *Tp53* and *RB*, in addition to *RAS*, *MYC*, and *BCL-2* oncogenes were identified in lung cancer. However, mutations of *Tp53* gene are occurring in 80% of SCLC and 50% of NSCLC. While *RB* mutations are detected in more than 90% of SCLCs. The identified molecular lung cancer biology are currently applying in development of a novel approaches in gene therapy of lung cancer (71).

1.6 MicroRNAs as biomarker of lung cancer

1.6.1 MicroRNAs overview

MicroRNAs (miRNAs) are a family of non-coding small RNAs, which consist of 19 to 25 nucleotides, and have main roles in regulation of gene expression at the posttranscriptional level by either degradation of mRNAs through base pairing to untranslated region (UTR) of the mRNAs molecule, or by inhibition the translation process by prevent the binding of ribosome to mRNAs molecule (72). Furthermore, it has been suggested that miRNAs target and regulate about 30% of human genes involved in various biological functions, including cellular proliferation, cell death, fat metabolism, and differentiation (73, 74).

Seventy percent of miRNA genes are mainly located within the exons of coding genes or within the introns while 30% are found in the intergenic regions which transcribed as independent to host genes (75).

1.6.2 MicroRNAs biogenesis

A stem-loop RNA structure, called the primary miRNA (pri-miRNA) which produced by RNA polymerase II or pol III enters two cleavage steps to generate the mature miRNA, one of them is nuclear and the other is cytoplasmic. However, these mechanisms are performed by two ribonuclease III endonucleases known as Drosha and Dicer (76). The first step of miRNA maturation process is nuclear cleavage of pri-miRNA (several Kilo bases) that possess 5 prime 7-methyl guanosine cap and 3 prime poly (A) tail into about 70 nucleotide long precursor miRNA (pre-miRNA) that has 5 prime phosphate and two nucleotide overhang at 3 prime end (Figure1.9). This process is accomplished by a protein complex composed of Drosha and the double-stranded RNA-binding protein DiGeorge syndrome critical region gene 8 (DGCR8) (73, 74, 76). Pre-miRNA assembles into Exportin-5 and RanGTP proteins; this complex prevents degradation of pre-miRNA and plays a critical role in the translocation into the cytoplasm. (77, 78). Cleavage of Pre-miRNA is achieved by Dicer and RNA-induced silencing complex (RISC). RISC is consisting of Argonaute 2 (Ago2) and transactivation-responsive RNA binding protein (TRBP). Ago2 cleaves 12 nucleotides at 3 prime end of the pre-miRNA generating Ago2-cleaved precursor miRNA (ac-pre-miRNA), then Dicer cleaves Ago2-

cleaved precursor miRNA to form miRNA duplex (76). Ultimately, a single stranded mature miRNA is obtained from unwinding of double stranded miRNA and degradation of passenger strand. The mature miRNA is combined with a protein complex called RISC and act as a guide for mRNA degradation (Figure 1.9) (79, 80).

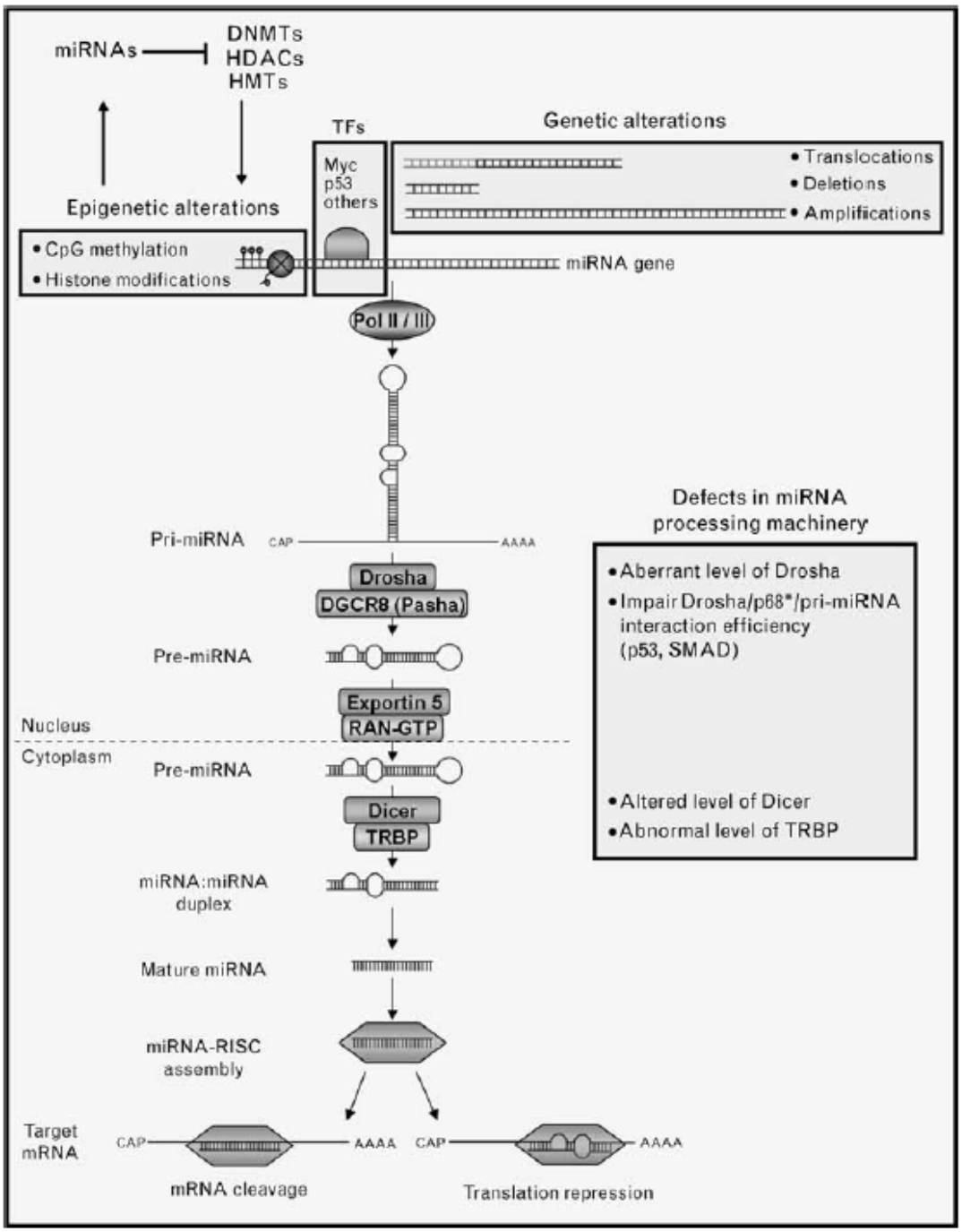


Figure 1.9 MicroRNA biogenesis and deregulation in cancer (81). DNMTs, DNA methyltransferases; HDACs, histone deacetylases; HMTs, histone methyltransferases; miRNAs, micro-RNAs; Pri-miRNA, primary microRNA; Pre-miRNA, precursor microRNA; RISC, RNA-induced silencing complex; TFs, transcription factors; TRBP, TAR RNA-binding protein.

1.6.3 MiRNAs target recognition

MiRNA-RISC assembly binds to the target mRNA transcript through base-pairing between the guide miRNA and 3’ UTR of the mRNA target. (82, 83). The target recognition strongly depends on the base-pairing with the miRNA seed (2-8 nucleotides at the 5’ end) which determine the gene silencing mechanism (84). In animals, the imperfect hybridization with bulges formation obtained due to the 100% complementary in the seed region which responsible for miRNA specificity and activity but not at the whole miRNA (83). However, two different silencing mechanisms defined as slicer-dependent and slicer-independent silencing are found. Both of these mechanisms leading to down-regulation of gene expression either by degrades of mRNA or inhibits the translation process (85). In the slicer-dependent silencing mechanism, the target mRNA is extensively base-paired with miRNA in the seed and 10 bases of the guide (86). The mRNA cleavage mechanism is catalyzed by Ago2 endonuclease. A protein complex consist of Pop2, Ccr4 and Not1 removes the poly (A) tail as the first step. Subsequent degradation occur either by the exosome (multi-protein complex with 3’ to 5’ exonuclease activity), or by Dcp1 and Dcp2 enzymes that undergo decapping of the mRNA which assists in 5’ to 3’ degradation by the Xrn1p exoribonuclease (Figure 1.10) (87).

The slicer-independent silencing mechanisms occur due to an imperfect base- pairing between the mRNA target and miRNA guide that make bulges, leading to inhibition of the activity of Ago2 slicer (82). Recent

studies suggest the inhibition of translation process by miRNA at the initiation and elongation steps depending on the mRNA promoter. Additionally, translation can be suppressed indirectly by spatial separation of translation components such as miRNA-mRNA duplex, leading to isolation of mRNA away from the ribosomes into cytoplasmic components known as P-bodies (88). Also, miRNA can accelerate deadenylation and dacapping processes which decrease the stability of the mRNA transcript. However, mRNA transcript could be stored or degraded by exosome or Xrn1p exoribonuclease (Figure 1.11) (89).

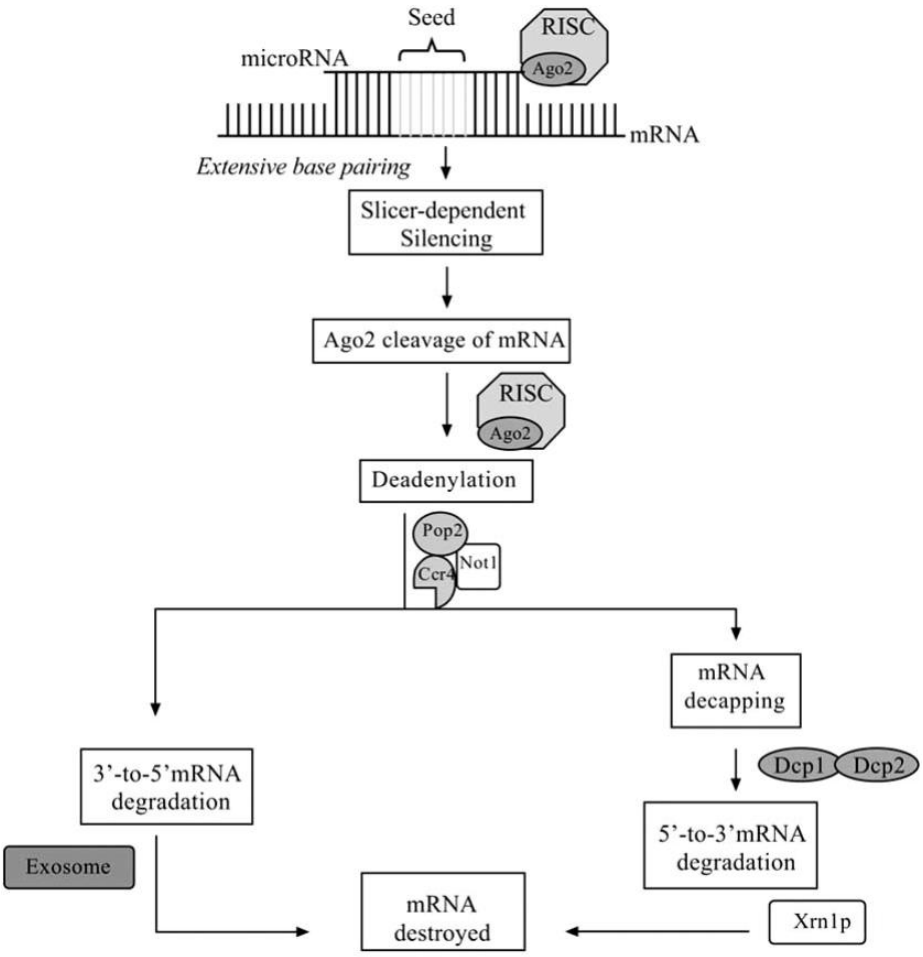


Figure 1.10 Slicer-dependent gene silencing mechanism (88).

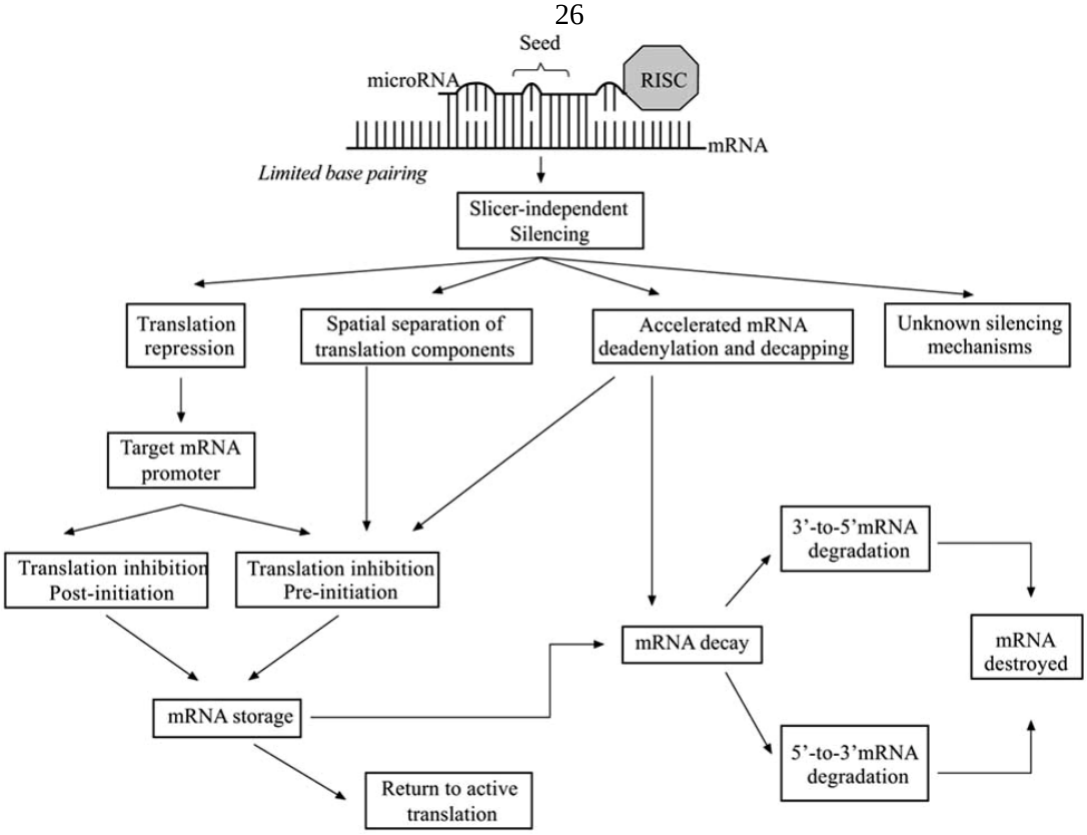


Figure 1.11 Slicer-independent gene silencing mechanism (88).

1.6.4 MiRNAs deregulation in lung cancer

Many studies have proven that miRNA expression alterations occur not only in many human diseases but also in cancer (90). In addition, deregulation of miRNA expression was observed in tumor tissues with variations according to tumor type, compared with normal tissues. Chromosomal abnormalities are considered the main genetic changes related to miRNA deregulation expression. It is noticed that about 50% of miRNA genes are found in a chromosomal fragile sites, this sites show breaks during DNA replication when the cell exposed to stress, which associated with cancer (80). Consequently, miRNA expression levels linked with alterations in copy number of any loci on genome.

MiRNAs play an important role in a tumor suppression, which contribute to the development of a malignant cell when loses its function by mutations, and genomic alterations (91). Tumor suppressor miRNAs play an essential role in prevention of tumor formation by suppressing oncogenes and anti-apoptotic genes. It may also act as oncogenes, these oncogenic miRNAs target tumor suppressor and apoptotic genes (92).

Let-7 family, a cluster of miRNAs was considered as tumor suppressor. The expression of *let-7* family were reduced in NSCLC patients and closely related to poor prognosis (93). Furthermore, a single nucleotide polymorphism was found in *let-7* complementary site 6 of 3 prime – UTR of K-RAS mRNA which strongly associated with NSCLC among smokers (94).

Overexpression of miR-17-92 cluster, which located in 13q31.3, has been observed in lymphomas and solid tumors as a result to amplification of this region (95).

Many studies were focused on miRNA deregulation expression in different types of cancer. Let-7 family, miR-9 family, miR-29 family, miR-148a, and miR-124a family were shown down-regulation in lung cancer. While miR-17-(to) 92 cluster, miR-21, and miR-155 have showed upregulation in lung cancer (96, 97, 98).

A study on 74 lung cancer patients and 68 cancer-free controls was investigated on 15 miRNAs including miR-17, 21, 24, 106a, 125b, 128, 155, 182, 183, 197, 199b, 203, 205, 210 and 221. As a result, this study showed

significant elevation of miR-155, miR-197, and miR-182 in the plasma of these patients compared with controls. The sensitivity and specificity were 81.33% and 86.76% respectively (99).

In 2011, a study on miR-21 as a biomarker for early detection of non-small cell lung cancer (NSCLC) was evaluated. The expression level of miR-21 on 63 NSCLC patients and 30 healthy controls was detected by real-time fluorescence quantitative PCR. PCR results showed significant elevation in the miR-21 expression in patients with advanced stage of NSCLC than in the group with early stage NCSLC and controls ($p < 0.001$). The sensitivity and specificity were 76.2% and 70.0%, respectively. Consequently, the study was suggested that miR-21 expression correlated to tumor progression factors, and it is effective marker for early diagnosis of NSCLC (98).

Another valuable study using sera of 130 subjects (55 NCSLC patients, and 75 healthy subjects) to examine the expression level of 181 miRNAs was done. All patients were matched for age, and smoking history. The study showed that miR-15b and miR-27b were able to distinguish NSCLC patients from healthy controls with a specificity of 84% (95% CI 0.73-0.91), sensitivity of 100% (95% CI 0.93-1.0), negative predictive value (NPV) of 100%, and a positive predictive value (PPV) of 82% (100).

At the level of metastasis, some miRNAs have been noticed as biomarkers of cancerous cells metastasis, which considered the main reason of mortality among cancer patients who have solid tumors.

1.6.5 Defects in microRNA biogenesis and lung cancer

Imperfection of miRNAs processing machinery may be related to oncogenesis. Reduction of Dicer, Drosha, and DGCR8 expression levels enhance tumor development due to decreasing of mature miRNA formation (101). A study by Karube *et al* has reported the correlation between poor survival rate and decreasing of Dicer expression level on 67 NSCLC patients who surgically resected (102).

1.6.6 Epigenetic causes of microRNAs deregulation in cancer

Epigenetic mechanisms including CpG island methylation and histone modification are also considered an important reason lead to deregulation of miRNA expression in cancer. However, many of miRNAs deregulated in several types of cancer, CpG hypermethylation and histone modification of miR-127 is revealed in bladder cancer (103) and miR-21, miR-205, and miR-203 in ovarian cancer (104).

Recent studies have shown that miRNAs may effect on modulation of DNA methylation and histone modifications. DNA methyltransferases (DNMTs), histone deacetylases (HDACs), and histone methyltransferases (HMTs) considered targets of several miRNAs. A study in 2009 was shown that miR-29b direct acts on DNA methyltransferase 3 alpha (DNMT3A) and DNA methyltransferase 3 beta genes (DNMT3B), global DNA hypomethylation has noticed due to decreasing in DNMTs expression that responsible for DNA methylation (105).

1.6.7 MicroRNAs as biomarker for cancer

Depending on several studies, miRNAs considered highly stable molecule, it is protected from RNases activity in the cell. This advantage allows efficient isolation and detection from different types of samples such as, sputum, serum, plasma, and also formalin-fixed paraffin embedded tissue, with multiple freeze-thawing cycles without disruption. Therefore, miRNAs can be used as noninvasive biomarkers for early detection, diagnosis and classification of human cancer (106).

1.6.8 miR-21

MiR-21 is 22 nucleotides located at 17q23.1. It is classified as an oncogene and considered as anti-apoptotic factor. Over-expression of miR-21 minimizes the expression of apoptotic genes (107). The over-expression of miR-21 not only observed in lung cancer but also in breast cancer, colon cancer, liver, and brain cancers (108).

Recent studies on miR-21 were shown that it affects and suppresses four tumor suppressor genes including mapsin, phosphatase and tensin homolg (PTEN), tropomyosin1 (TPM1), and programmed cell death 4 (PDCD4) (Figure 1.12). MiR-21 prevent the translation of these four genes

by binding to their UTR of the transcript. The results are tumor growth, cell transformation, and metastasis (109, 110, 111).

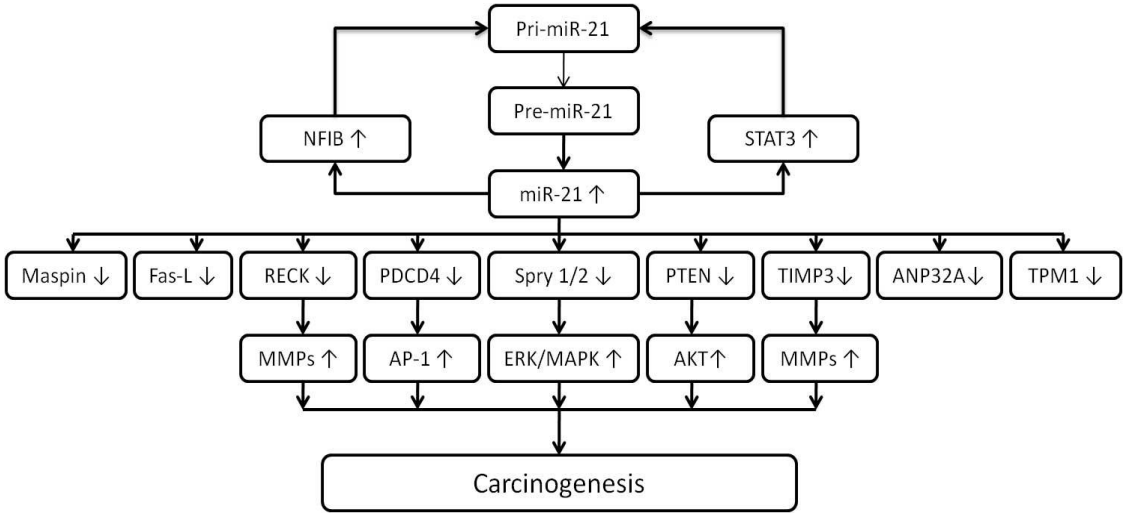


Figure 1.12 Pathways of miR-21 in cancer development (126).

PTEN is a tumor suppressor that dephosphorylates PIP3 to obtain PIP2 in conjunction with phosphoinositide 3-kinase (PI3K), which in turn phosphorylates PIP2 into PIP3, the result is maintaining the balance of PIP3 levels that regulate AKT pathway. Translation suppression of PTEN occur by miR-21 leads to accumulation of high concentration of PIP3 which activate the AKT pathway that stimulate cell growth and survival (Figure 1.13) (112).

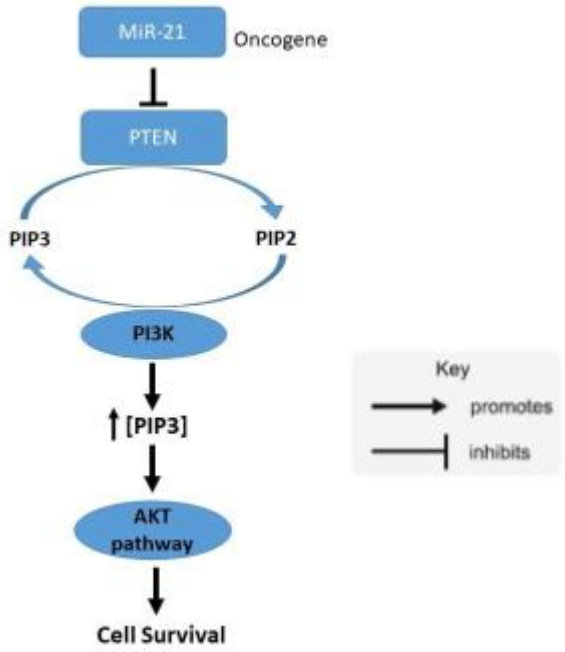


Figure 1.13 MiR-21 and its impact on signaling pathway of cell survival.

1.6.9 miR-15b

Mir-15b is one of the four members of miR15/16 family which described as tumor suppressor. All four members are organized in two different clusters; the first one comprises miR15a/miR16-1, while the second cluster includes miR15b/miR16-2 (113, 114). MiR15/16 family has a role in the post-transcriptional suppression by targeting and sharing 9-nucleotide seed region at 3’ UTR of oncogenic anti-apoptotic BCL2 protein. Over-expression of the anti-apoptotic BCL2 protein was observed in many different tumors which leads to cell survival (115, 116).

MiR-15b is a 22 nucleotides located at 3q25.33 and considered as tumor suppressor. BCL2 has been investigated to be a target gene of several miRNAs such as miR-15b through targeting 3’ UTR of BCL2 mRNA (117). BCL2 protein functions to cell survival by repressing BAX protein and

inhibiting releasing of mitochondrial cytochrome C which in turn activates caspase pathway responsible for apoptosis. Over-expression of miR-15b leading to inhibition of BCL2 protein and activating of intrinsic pathway of programmed cell death (Figure1.14) (118). Upregulation of miR-15b in NSCLC especially lung adenocarcinoma cells was reported which promote cellular proliferation and invasion (119).

A study on expression of miR-15b in NSCLC was demonstrated the effect of this microRNA on tissue inhibitor metallopeptidases 2 (TIMP2) protein. The results of this study were shown that TIMP 2 is a target of miR-15b and it was downregulated as a result of miR-15b upregulation which promotes the development, proliferation, and invasion of NSCLC (119).

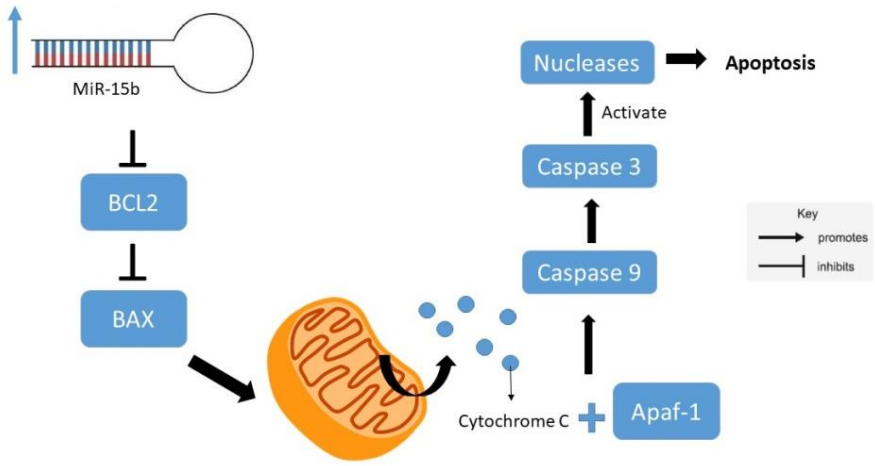


Figure 1.14 Over-expression of miR-15b which represses BCL2 anti-apoptotic protein and induces intrinsic apoptosis pathway.

Table 1.1 miRNAs type, chromosomal location, expression in human lung cancer, and sequence of mature form.

MiRNA	Gene type	Chromosomal location	Expression in lung cancer	Mature miRNA sequence
miR-21	Oncogene	17q23.1	Increasing	5'- UAGCUUAUCAGACUGAUGUUGA -3'
miR-15b	Tumor suppressor	3q25.33	Increasing	5'- UAGCAGCACAUCAUGGUUUACA -3'

1.7 objectives

According to studies that concern on miRNA expression levels as a biomarker for detection of lung cancer. The results qualify them to be valuable biomarkers for early detection of lung cancer for high risk people who work in downtown transportation center in Nablus.

1- Evaluation the validity of the downtown transportation center in Nablus for people working.

Study the probability of lung cancer development for these people since the ventilation of this transportation center is insufficient, which lead to high accumulation level of diesel exhaust emissions.

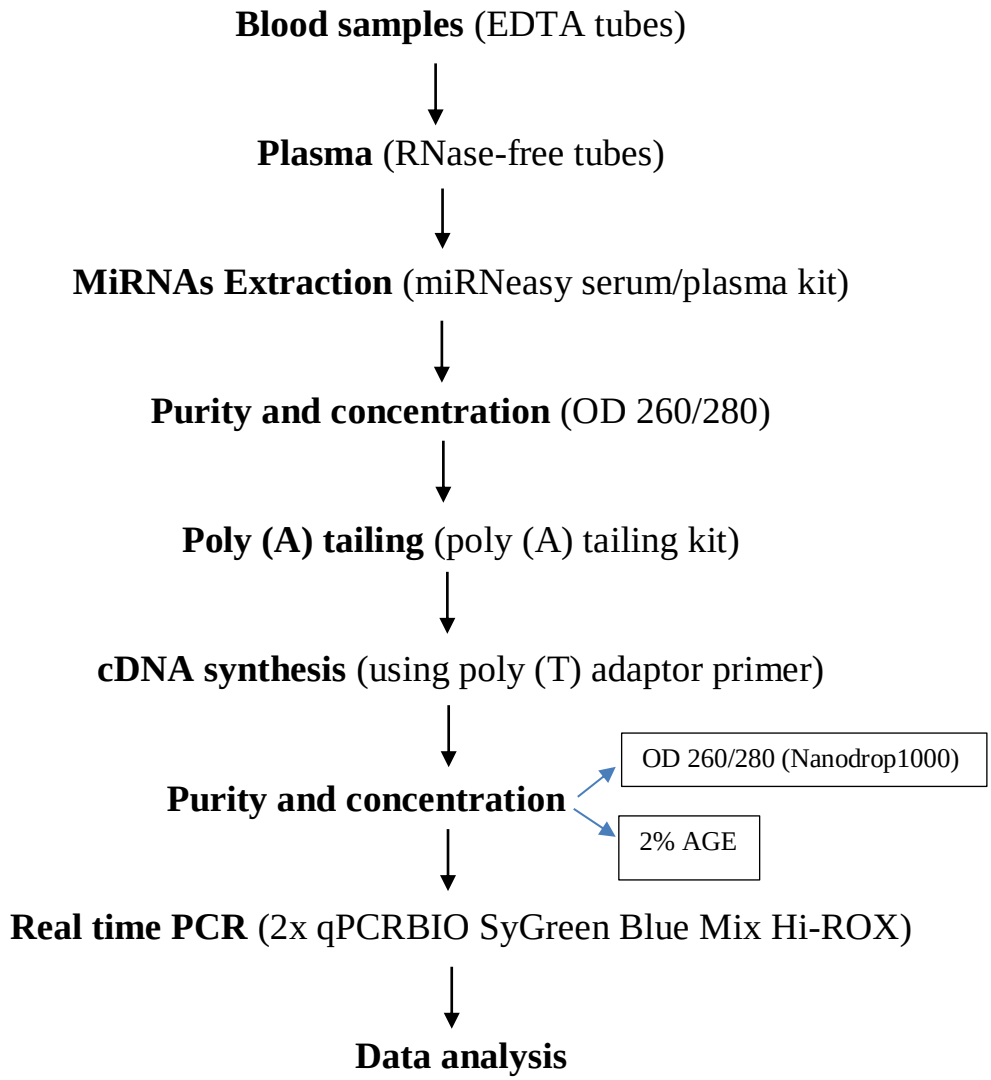
2- Identification of biomarkers for early detection of lung cancer in Palestine.

The identification of non-invasive and cost effective diagnostic test for early detection of lung cancer and use it as a first line screen to help risk people.

Chapter Two

Materials and Methods

2.1 Summary of Methods



2.2 Study population

The study population was 20 samples; divided into three groups:

1) Group (1): 10 samples from nonsmokers people working at downtown transportation center in Nablus for at least 4 hours of daily exposure to diesel exhaust emissions for long time.

2) Group (2): 5 samples from nonsmoker's healthy people who do not exposed to diesel exhaust emissions for long time (cancer-free control).

3) Group (3): 5 samples from lung cancer patients who are under treatment at An-Najah National University Hospital.

2.3 Permission and ethical consideration

According to research ethics, permission was obtained from Institutional Review Board (IRB) and Ministry of Health (MOH). The approval was obtained and the objectives of the study were also clarified for all participants.

2.4 Materials

2.4.1 Reagents and Chemicals

The list below contains the reagents and chemicals used in the study:

- MiRNeasy serum/plasma kit (Qiagen)
- MiRNeasy serum/plasma kit spike-in control (Qiagen)

- 2x qPCRBIO SyGreen Blue Mix Hi-ROX kit (PCR biosystems)
- UltraScript Reverse Transcriptase kit (PCR biosystems)
- Poly(A) tailing kit (Ambion)
- Absolute Ethanol
- DNA ladder, 100bp
- Ultra-pure Agarose
- Gel Red
- Chloroform
- TBE buffer

2.4.2 Disposables

The major disposables are mentioned in the list below:

- 1.5 ml RNase free microcentrifuge tubes
- RNase free micropipette tips
- Disposable gloves
- 0.2 ml PCR micro tubes
- RNase free water
- 4 ml EDTA tubes

- Disposable syringes and needles

2.4.3 Equipment's

The experiments of the study were achieved in the research laboratory at department of biology and biotechnology, An-Najah National University. The major equipment's are mentioned in the list below:

- Biosafety cabinet
- Microcentrifuge
- Thermocycler
- Vortex mixer
- Micropipette
- Electrophoresis
- UV-Transilluminator
- Refrigerator (-20 °C)
- Deep freeze (-80 °C)
- Microwave
- Autoclave
- Real-time PCR (Bio-rad CFX Maestro)

2.5 Methods

2.5.1 People selection

Selection of high risk people who work at the downtown transportation center in Nablus according to the following criteria:

- Nonsmokers
- Duration of diesel exhaust emissions (long time of exposure to diesel exhaust emissions for at least 4 hours daily for more than 5 years)
- Free cancer history

2.5.2 Blood samples collection and plasma preparation

Peripheral blood samples (5 ml) were collected from 20 individuals. Clinical information were collected from each individual using a questionnaire; the data includes cancer history, smoking history, duration of diesel exhaust exposure, and exposure intensity. All smokers will be excluded from the study. Fresh whole blood samples in EDTA preservative were centrifuged immediately at 1900 g on 4 °C for 10 minutes. Plasma were collected (upper yellow phase) to a new RNase-free tubes. Plasma samples were centrifuged on 1600 g at room temperature for 10 minutes. Supernatants were transferred to a new RNase-free tubes and stored at -80 °C until use.

2.5.3 Plasma miRNA extraction

Plasma miRNA was extracted using Qiagen miRNeasy serum/plasma kit that combines phenol/guanidine–based lysis of samples and silica-membrane-based purification of total RNA including miRNAs according to manufacturer's protocol as follows:

1) 1000 µl of QIAzol lysis reagent were added to 200 µl plasma in 1.5 ml RNase-free centrifuge tube then tubes were incubated after pipetting up and down on the benchtop at room temperature for 5 minutes.

2) 3.5 µl of miRNeasy serum/plasma spike-in control (from $1.6x10^8$ copies/µl working solution) were added and mixed by pipetting.

3) 200 µl of chloroform were added then shaken vigorously for 15 seconds and incubated at room temperature for 3 minutes.

4) The tubes were centrifuged at 12000 g on 4 °C for 15 minutes.

5) Upper aqueous phase (600 µl) was transferred to a new RNase-free collection tubes then 900 µl of 100% ethanol were added (total volume is 1.5 ml) and mixed by pipetting.

6) 700 µl of solution were pipetted up and transferred to RNeasy MinElute spin column in a 2 ml collection tube. The led was closed and tubes were centrifuged at 8000 g for 15 seconds at room temperature then the flow-through was discarded.

7) The remained solution (800 µl) were recollected into the same RNeasy MinElute spin column and collection tube. Also, tubes were centrifuged at 8000g for 15 seconds at room temperature then the flow-through was discarded.

8) 700 µl of RWT buffer were added to the RNeasy MinElute spin column then centrifuged at 8000 g for 15 seconds. The flow-through was discarded.

9) 500 µl of RPE buffer were added to the RNeasy MinElute spin column and also tubes were centrifuged at 8000g for 15 seconds. The flow-through was discarded.

10) 500 µl of 80% ethanol were added to the column then tubes were centrifuged at 8000 g for 2 minutes and the collection tubes with the flow-through were discarded.

11) RNeasy MinElute spin columns were placed into a new 2ml collection tubes. The lid of spin columns were opened and centrifuged at full speed for 5 minutes to dry the membrane. Then the collection tubes and the flow-through were discarded.

12) 14 µl of RNase-free water were added directly to the center of RNeasy MinElute spin columns. Tubes with spin columns were centrifuged at full speed for 1 minutes. The result was 12 µl elute containing miRNAs.

2.5.4 MiRNAs purity and concentration

The purity and concentration of extracted miRNA samples were determined by NanoDrop 1000.

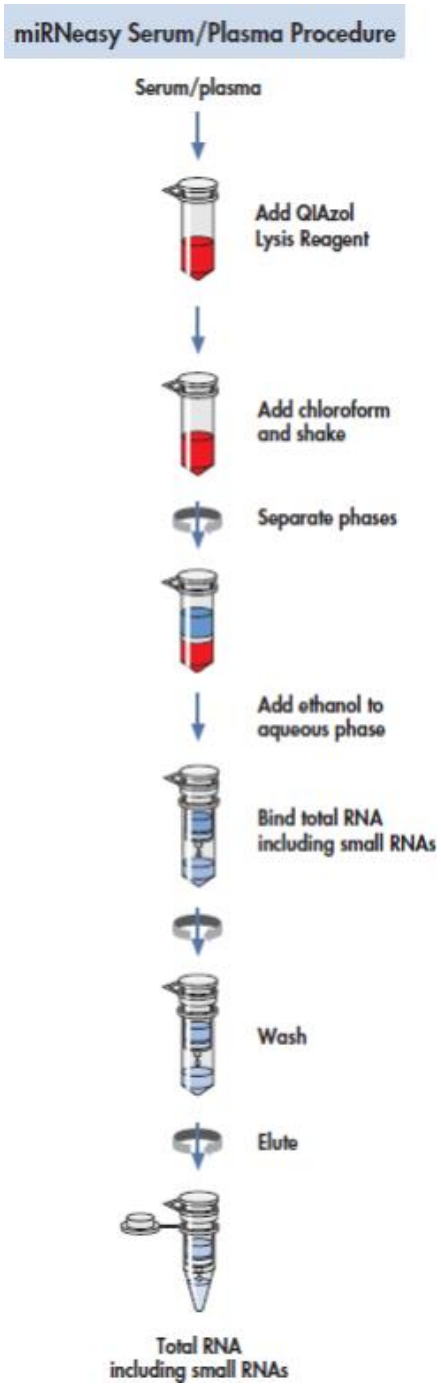


Figure 2.1 MiRNAs extraction protocol summary.

2.5.5 Poly (A) tailing of miRNAs

Less than 150 adenine base were added to the miRNAs using the poly (A) tailing kit (Applied Biosystems) according to manufacturer's protocol as follows:

1) 25 µl total volume tailing reactions were obtained by adding reagents as mentioned in the following table:

Table 2.1 Poly (A) tailing reaction components.

Component	Amount	Final concentration
5x *E*-PAP Buffer	5 µl	1x
Nuclease-free Water	11 µl	
25mM $MnCl_2$	2.5 µl	2.5mM
10mM ATP	2.5 µl	1mM
MiRNA template (40ng/µl) (or spike-in control)	3 µl	
E-PAP Enzyme (2 units/µl)	1 µl	0.08 units

2) All tubes were incubated at 37 °C for 1 hour and then stored at -20 °C.

2.5.6 cDNA synthesis

Reverse transcription reaction was achieved using UltraScript Reverse Transcriptase kit (PCR Biosystems) with a poly (T) adapter (5'GCGAGCACAGAATTAATACGACTCACTATAGGTTTTTTTTTTT TTTTVN-3'). This kit was modified to enhance cDNA synthesis and yield using manipulated MMLV reverse transcriptase. The reaction was done according to manufacturer's protocol as follows:

1) 5x UltraScript buffer was briefly vortexed and thawed.

2) Master mix was prepared with 20 µl total volume as shown in the following table:

Table 2.2 cDNA synthesis reaction mixture.

Component	Amount	Final concentration
5x UltraScript Buffer	4 µl	1x
Nuclease-free Water	9 µl	
UltraScript (200units/µl) (with RNAse inhibitor)	1 µl	
Poly(A) tailed miRNAs	4 µl	
10x Oligo-dT adaptor primer	2 µl	1x

3) All tubes were incubated at 42 °C for 30 minutes then at 85 °C for 10 minutes.

4) $1x10^6$ copies/µl of miRNeasy serum/plasma Spike-In control concentration was obtained by adding 28 µl of RNase-free water to the cDNA reactions.

5) All tubes were stored at -20 °C until use.

2.5.7 cDNA check

Synthesized cDNA was checked on 2% agarose gel using ultrapure agarose (Qiagen) and TBE buffer.

2.5.8 cDNA purity and concentration

Purity and concentration of cDNA samples were calculated using Nanodrop 1000.

2.5.9 MicroRNAs quantification by real-time PCR

2.5.9.1 Real-time PCR Overview

In contrast to conventional PCR, real-time PCR is a technique which can collect data while PCR process progresses. Real-Time detection of products is achieved by presence fluorescent molecules that reports an elevation of DNA quantity which correlated to fluorescence intensity. The advantage of real-time PCR represented in ability to determination the starting copy number of the DNA template with high accuracy (122).

In real-time PCR amplification plot, the PCR cycle number is shown on the x-axis, while y-axis shown the fluorescence which proportional to the quantity of the amplified DNA. Two phases are found in amplification plot, an exponential phase which related to doubles the PCR product amount in each cycle. The plateau phase started when the reaction components are expended (Figure 2.2).

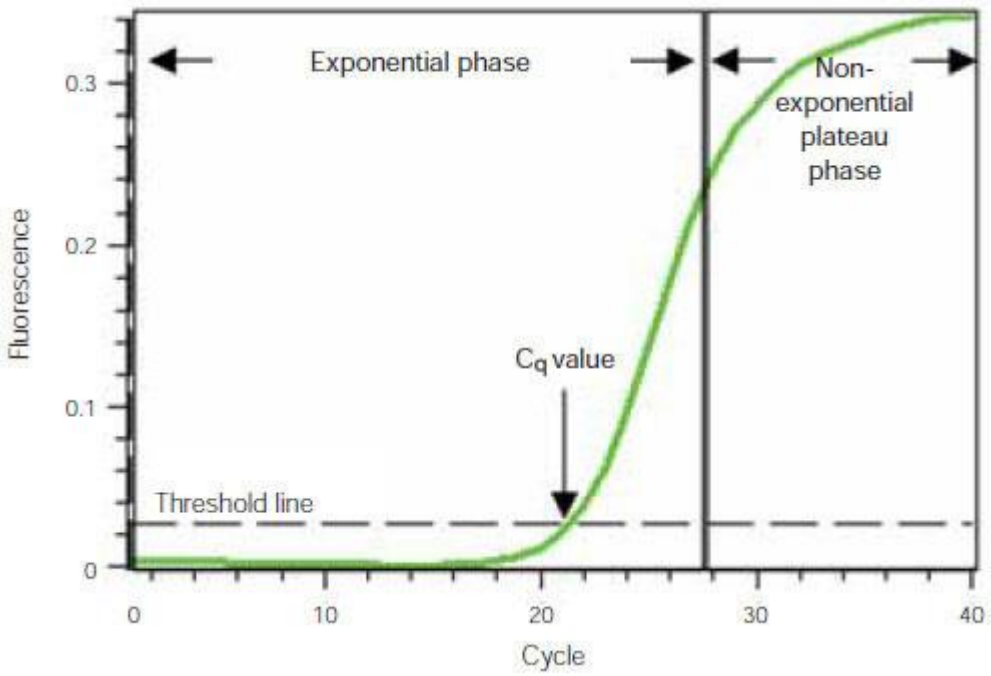


Figure 2.2 Real-time PCR amplification plot (Real-time PCR applications guide, Biorad).

2.5.9.2 DNA binding dyes (SYBR Green l)

SYBR Green l is a nonspecifically double stranded DNA binding dye used in real-time PCR which shows a fluorescence when binds to dsDNA. The fluorescence proportional to the amount of the DNA product in the tube, since it increases when the product is amplified (Figure 2.3).

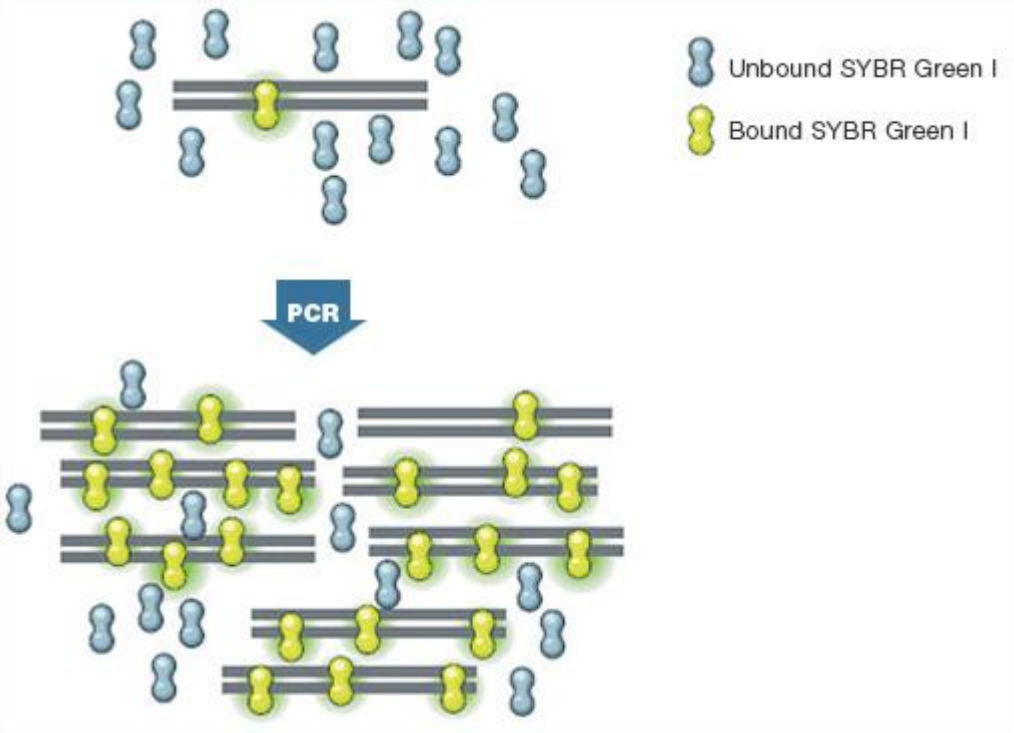


Figure 2.3 SYBR Green l in real-time PCR shows increases in fluorescence when the dye binds to double stranded DNA (Real-time PCR applications guide, Biorad).

There are many advantages of using DNA-binding dyes over probes represented in:

1- Simple design of the assay (only two primers).

2- Lower costs.

3- Ability to make melt-curve analysis for specificity check.

2.5.9.3 Real-time PCR protocol

A SYBR Green-based quantitative real time PCR assay was used for miRNA expression quantification. Real-time PCR was carrying out using 2x qPCRBIO SyGreen Blue Mix Hi-ROX (PCR Biosystems) with specific forward primers and universal reverse primers, which were complementary sequence to poly (T) adapter primer.

Real-time PCR reaction was performed in 20 µl total volume for miR-21 and miR-15b as follows table:

Table 2.3 Real-time PCR reaction components.

Component	Amount	Final concentration
2x qPCRBIO SyGreen Blue Mix	10 µl	1x
Forward primer (10µM)	0.8 µl	400nM
Universal reverse primer (10µM)	0.8 µl	400nM
Template cDNA	2 µl	
PCR grade dH_2O	6.4 µl	

The real time PCR assay was performed in Bio-rad CFX system in duplicates as following steps: initial denaturation at 95 °C for 2 minutes, then 50 cycles of 95 °C for 5 seconds and 60 °C for 30 seconds (Figure 2.4).

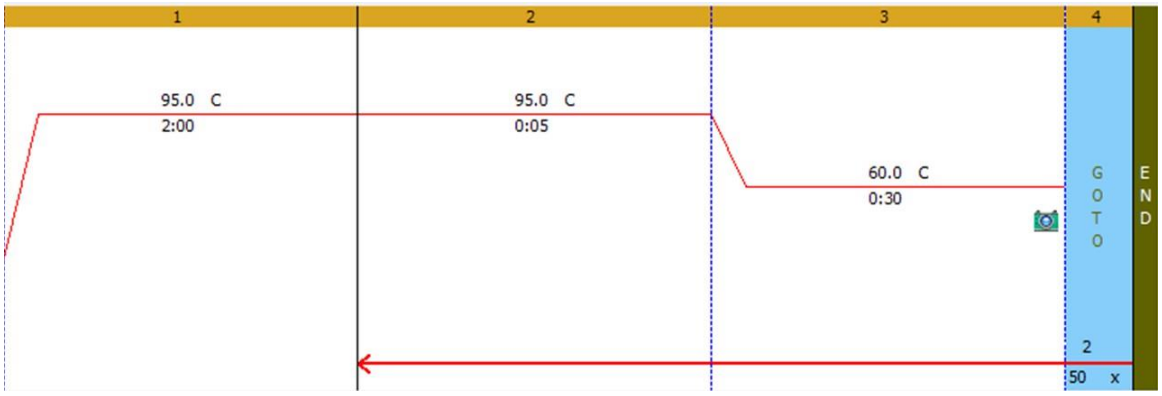


Figure 2.4 Real-time PCR run program.

2.5.9.4 Annealing temperature optimization

The detection of the optimal annealing temperature of the primers was achieved by a separated reaction using a range of annealing temperatures above and below the T_m of the primers. The gradient temperatures were shown in figure 2.5. The optimal temperature was 60 °C.

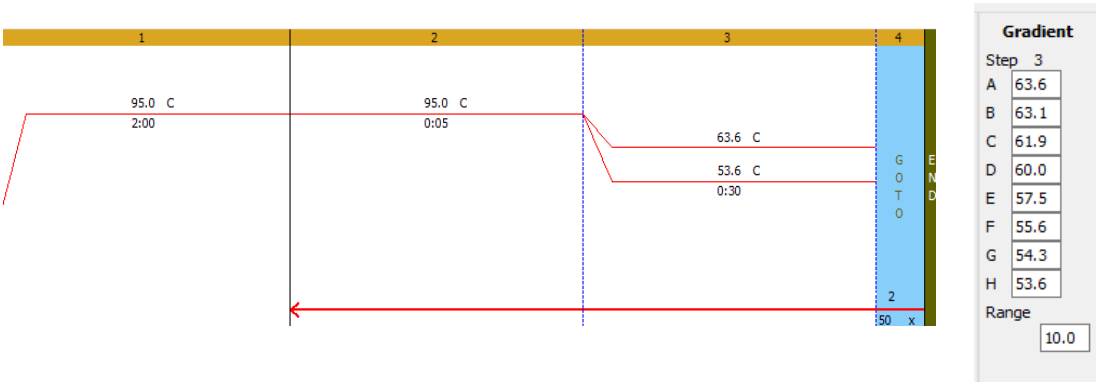


Figure 2.5 Gradient real-time PCR run program showing gradient temperatures for optimum annealing temperature.

2.5.9.5 Generation of standard curve for absolute quantification of miRNAs

Spike-in control (Ce_miR-39) was poly adenylated and reverse transcribed to cDNA as follows:

1) $1x10^8$ copies/µl of spike-in control working solution was prepared from stock solution.

2) Poly (A) tailing was done as in table 2.1. The final concentration of spike-in control was $1.2x10^7$ copies/µl.

3) Reverse transcription of poly (A) tailed Ce_miR-39 spike-in control was done as in table 2.3. The final concentration of spike-in control was $2.4x10^6$ copies/µl.

4) 28 µl of RNase-free water was added to the cDNA samples. The final concentration of spike-in control was $1x10^6$ copies/µl.

Serial dilutions of spike-in control (Ce_miR-39) cDNA were prepared for standard curve generation as mentioned in (Table 2.4). Standard curve was used for estimation of the recovery of spike-in control which was added to the plasma samples.

Table 2.4 Serial dilutions of spike-in control (Ce_miR-39) cDNA for standard curve generation.

Tube	cDNA	Water	Spike-in control concentration	Use in PCR
1	20 µl diluted cDNA	20 µl	5 x 10^5 copies/µl	2 µl (1 x 10^6 copies)
2	5 µl from tube 1	45 µl	5 x 10^4 copies/µl	2µl (1 x 10^5 copies)
3	5 µl from tube 2	45 µl	5 x 10^3 copies/µl	2 µl (1 x 10^4 copies)
4	5 µl from tube 3	45 µl	5 x 10^2 copies/µl	2 µl (1 x 10^3 copies)

5) Real-time PCR for spike-in control was performed as mentioned in (Table 2.3).

6) Data were analyzed using Bio-Rad CFX Maestro system and SPSS software for statistical analysis.

Table 2.5 The sequences of primers.

Primer	Sequence
Universal reverse primer	5’ – GCGAGCACAGAATTAATACGACTCA -3’
miR-15b (forward)	5’ –TAGCAGCACATCATGGTTTACA -3’
miR-21 (forward)	5’ -TAGCTTATCAGACTGATGTTGA -3’

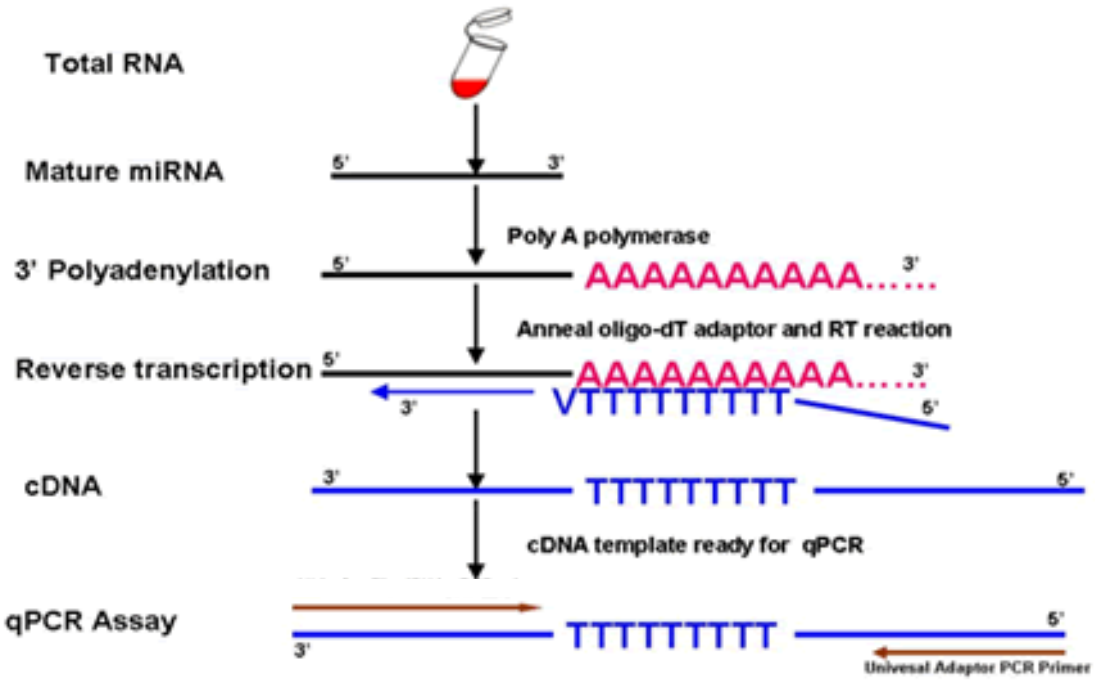


Figure 2.6 Overview of miRNAs detection steps including polyadenylation, reverse transcription, and quantitative PCR assay (120).

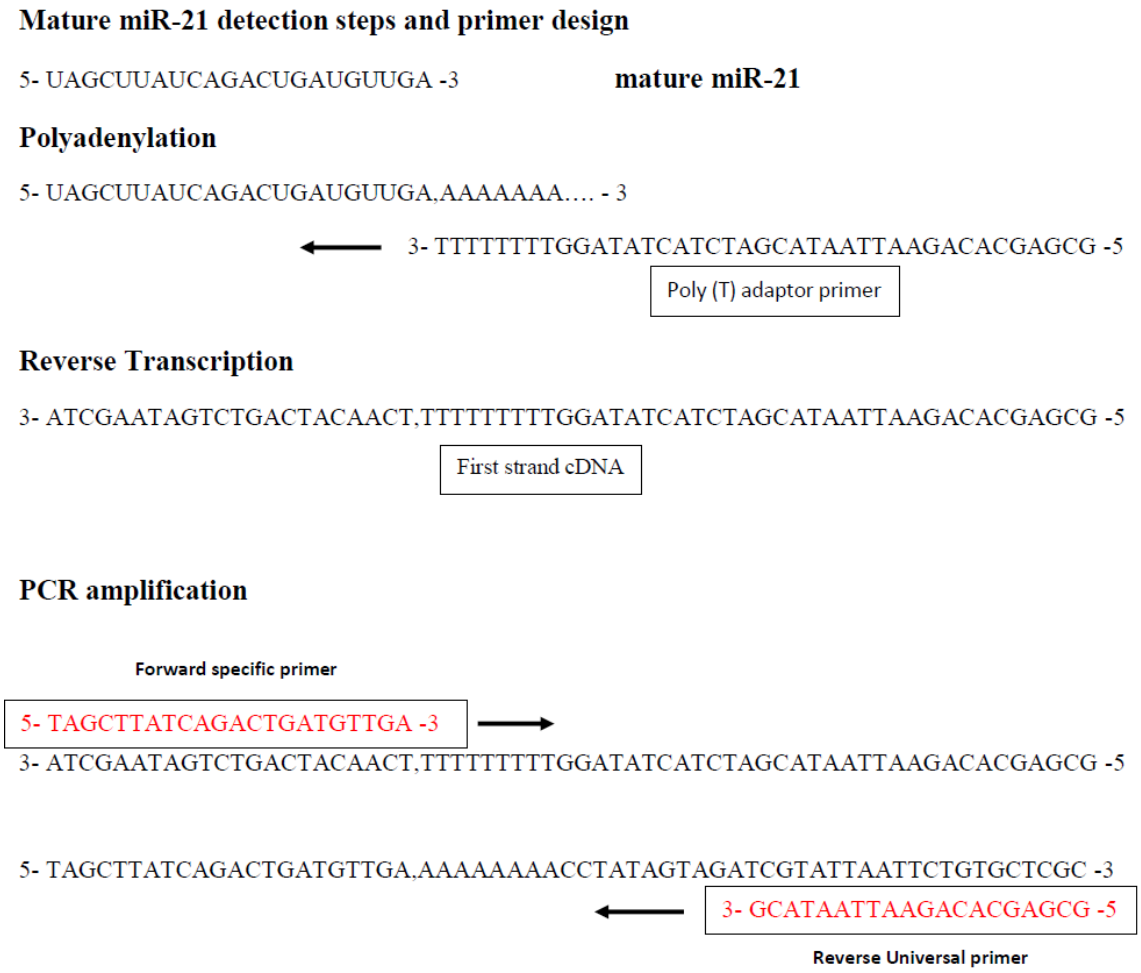


Figure 2.7 MiR-21 detection steps and primer design.

Figure 2.8 MiR-15b detection steps and primer design.

Chapter 3

Results and discussion

3.1 MiRNAs purity and concentration

Purity and concentration of extracted miRNAs were calculated using Nanodrop 1000. Purity was calculated by optical density of sample using the equation OD_{260}/OD_{280}. The results were shown in table as follows:

Table 3.1 Purity and concentration of miRNA plasma extracted samples. Samples from 1 to 10 were classified as DME exposed samples. Samples from 11 to 15 were classified as healthy (Cancer-free control). Samples from 16 to 20 were classified as lung cancer patients (+ve control).

Sample number	(A_{260}/A_{280}) purity	miRNA concentration (ng/µl)
1	1.61	49.2
2	1.48	41.8
3	1.60	133.4
4	1.64	165.8
5	1.58	56.3
6	1.55	54
7	1.54	50.6
8	1.55	133.2
9	3.71	279.2
10	1.65	61.1
11	1.66	95.6
12	1.57	72.8
13	1.62	158
14	1.48	117.1
15	1.42	112.9
16	1.51	64.6
17	1.48	105.4
18	1.46	89
19	1.52	83.2
20	1.44	54.1

Based on the results of the above table, spectrophotometric quantification of RNA (A_{260}) and RNA purity (A_{260}/A_{280}) results were

obtained for extracted RNA including miRNA. The concentration of all miRNAs is good.

3.2 cDNA check

Purity was calculated by optical density of sample using the equation OD_{260}/OD_{280}. The mean of all cDNA samples concentration was 221.13 ng/µl.

Table 3.2 The concentration and purity of cDNA samples using Nanodrop 1000.

Sample number	(A_{260}/A_{280}) purity	cDNA concentration (ng/µl)
1	2.51	347.8
2	2.32	259.4
3	2.42	259.5
4	2.56	301.6
5	2.31	211.3
6	2.38	373.7
7	1.88	249.6
8	2.12	251
9	2.01	131.5
10	2.16	187.3
11	2.15	192.4
12	1.79	231.8
13	1.91	208
14	1.99	210.2
15	1.91	145.2
16	1.85	223.3
17	1.97	166.8
18	1.86	186.2
19	1.81	121.7
20	1.87	164.3

3.3 MicroRNAs Absolute quantification by real-time PCR

3.3.1 Standard curve analysis

A standard curve was constructed from 10-fold dilutions of a known concentration cel_mir-39 spike-in control, the x-axis shows the logarithm of the initial copy number, while C_T values plotted along the y-axis. The results were obtained as follows:

- Efficiency = 104.6 % (100% efficiency means the template doubles after each cycle).
- Correlation coefficient (R^2) = 0.995 (how will the data fit the standard curve).
- Slope = -3.216 (measure the reaction efficiency).
- y-intercept = 46.055 (related to the C_T value expected if the lowest copy number of the target molecule).

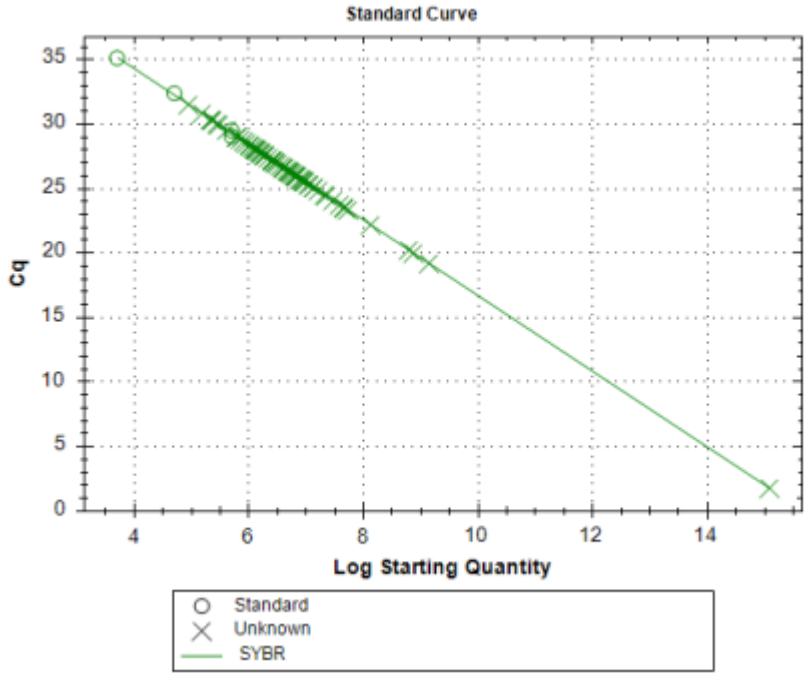


Figure 3.1 Standard curve. Cq values along the y-axis, the logarithm of the starting quantity of DNA along x-axis.

Depending on the standard curve that generated, the starting quantity of the target DNA in the unknown samples was estimated by interpolation. The expression of miR-21 and miR-15b was also estimated (Table 3.3).

3.3.2 Annealing temperature optimization

An annealing temperature gradient (from 53.6 °C to 63.6 °C) shows that the 60 °C is the optimal annealing temperature and was selected for the assay since it is the lowest C_T value compared to other temperatures.

3.3.3 Starting quantity of both miR-21 and miR-15b using real-time PCR

Table 3.3 Cq values, starting quantity, and expression of miR-21 and miR-15b of DME exposed samples (samples from 1 to 10), healthy control (samples from 10 to 15), and lung cancer patients control (samples from 16 to 20) using real-time PCR. Standard (Std) was obtained using Cel_miR-39 spike in control and non-template control (NTC) also showed. All samples are run in qPCR in duplicates (showed as prime (')).

Target	Content	Sample	Cq	Starting Quantity (SQ)	Log Starting Quantity	Expression
miR-21	DME exposed	1	28.69	$8.23*10^5$	5.915	Up-regulation
miR-21	DME exposed	1'	27.62	$19.03*10^5$	6.279	Up-regulation
miR-21	DME exposed	2	27.77	$17.01*10^5$	6.231	Up-regulation
miR-21	DME exposed	2'	26.03	$66.28*10^5$	6.821	Up-regulation
miR-21	DME exposed	3	29.05	$6.24*10^5$	5.795	Up-regulation
miR-21	DME exposed	3'	28.56	$9.16*10^5$	5.962	Up-regulation
miR-21	DME exposed	4	27.64	$18.75*10^5$	6.273	Up-regulation
miR-21	DME exposed	4'	28.09	$13.23*10^5$	6.122	Up-regulation
miR-21	DME exposed	5	28.37	$10.63*10^5$	6.026	Up-regulation
miR-21	DME exposed	5'	29.03	$6.33*10^5$	5.801	Up-regulation
miR-21	DME exposed	6	26.67	$40.35*10^5$	6.606	Up-regulation
miR-21	DME exposed	6'	26.96	$32.12*10^5$	6.507	Up-regulation
miR-21	DME exposed	7	29.56	$4.16*10^5$	5.619	Normal
miR-21	DME exposed	7'	30.37	$2.21*10^5$	5.345	Normal
miR-21	DME exposed	8	1.79	1210240192 $3.56*10^5$	15.083	Up-regulation
miR-21	DME exposed	8'	20.01	$7466.18*10^5$	8.873	Up-regulation
miR-21	DME exposed	9	22.20	$1338.94*10^5$	8.127	Up-regulation
miR-21	DME exposed	9'	20.25	$6187.92*10^5$	8.792	Up-regulation

miR-21	DME exposed	10	28.40	$10.36*10^5$	6.015	Up-regulation
miR-21	DME exposed	10‘	27.59	$19.55*10^5$	6.291	Up-regulation
miR-21	Healthy	11	30.84	$1.53*10^5$	5.185	Normal
miR-21	Healthy	11‘	30.30	$2.33*10^5$	5.367	Normal
miR-21	Healthy	12	28.07	$13.39*10^5$	6.127	Up-regulation
miR-21	Healthy	12‘	27.97	$14.50*10^5$	6.161	Up-regulation
miR-21	Healthy	13	31.53	$0.89*10^5$	4.948	Normal
miR-21	Healthy	13‘	29.04	$6.29*10^5$	5.799	Up-regulation
miR-21	Healthy	14	29.97	$3.02*10^5$	5.480	Normal
miR-21	Healthy	14‘	30.38	$2.20*10^5$	5.342	Normal
miR-21	Healthy	15	27.42	$22.32*10^5$	6.349	Up-regulation
miR-21	Healthy	15‘	28.06	$13.51*10^5$	6.131	Up-regulation
miR-21	Lung cancer	16	25.69	$86.60*10^5$	6.938	Up-regulation
miR-21	Lung cancer	16‘	30.06	$2.82*10^5$	5.450	Normal
miR-21	Lung cancer	17	26.37	$50.87*10^5$	6.706	Up-regulation
miR-21	Lung cancer	17‘	25.73	$84.21*10^5$	6.925	Up-regulation
miR-21	Lung cancer	18	23.53	$474.11*10^5$	7.676	Up-regulation
miR-21	Lung cancer	18‘	23.36	$539.70*10^5$	7.732	Up-regulation
miR-21	Lung cancer	19	24.11	$299.59*10^5$	7.477	Up-regulation
miR-21	Lung cancer	19‘	25.11	$137.28*10^5$	7.138	Up-regulation
miR-21	Lung cancer	20	26.67	$40.18*10^5$	6.604	Up-regulation
miR-21	Lung cancer	20‘	26.39	$50.17*10^5$	6.700	Up-regulation
miR-15b	DME exposed	1	27.22	$26.12*10^5$	6.417	Up-regulation
miR-15b	DME exposed	1‘	26.63	$41.69*10^5$	6.620	Up-regulation
miR-15b	DME exposed	2	23.78	$388.74*10^5$	7.590	Up-regulation
miR-15b	DME exposed	2‘	25.98	$69.27*10^5$	6.841	Up-regulation
miR-15b	DME exposed	3	26.09	$63.58*10^5$	6.803	Up-regulation
miR-15b	DME exposed	3‘	25.65	$89.77*10^5$	6.953	Up-regulation

miR-15b	DME exposed	4	24.47	$225.99*10^5$	7.354	Up-regulation
miR-15b	DME exposed	4‘	26.19	$58.58*10^5$	6.768	Up-regulation
miR-15b	DME exposed	5	25.58	$94.38*10^5$	6.975	Up-regulation
miR-15b	DME exposed	5‘	25.98	$69.66*10^5$	6.843	Up-regulation
miR-15b	DME exposed	6	25.80	$79.58*10^5$	6.901	Up-regulation
miR-15b	DME exposed	6‘	27.06	$29.75*10^5$	6.473	Up-regulation
miR-15b	DME exposed	7	24.55	$211.92*10^5$	7.326	Up-regulation
miR-15b	DME exposed	7‘	24.43	$228.03*10^5$	7.358	Up-regulation
miR-15b	DME exposed	8	25.32	$116.21*10^5$	7.065	Up-regulation
miR-15b	DME exposed	8‘	28.14	$12.69*10^5$	6.104	Up-regulation
miR-15b	DME exposed	9	19.22	$13871.9*10^5$	9.142	Up-regulation
miR-15b	DME exposed	9‘	27.83	$16.23*10^5$	6.210	Up-regulation
miR-15b	DME exposed	10	25.56	$96.34*10^5$	6.984	Up-regulation
miR-15b	DME exposed	10‘	23.57	$457.08*10^5$	7.660	Up-regulation
miR-15b	Healthy	11	30.09	$2.45*10^5$	5.39	Normal
miR-15b	Healthy	11‘	30.31	$2.31*10^5$	5.364	Normal
miR-15b	Healthy	12	30.41	$1.74*10^5$	5.241	Normal
miR-15b	Healthy	12‘	30.52	$1.05*10^5$	5.023	Normal
miR-15b	Healthy	13	29.91	$3.31*10^5$	5.52	Normal
miR-15b	Healthy	13‘	30.02	$3.01*10^5$	5.48	Normal
miR-15b	Healthy	14	27.07	$29.42*10^5$	6.469	Up-regulation
miR-15b	Healthy	14‘	27.18	$27.01*10^5$	6.432	Up-regulation
miR-15b	Healthy	15	28.24	$11.72*10^5$	6.069	Up-regulation
miR-15b	Healthy	15‘	25.99	$68.37*10^5$	6.835	Up-regulation
miR-15b	Lung cancer	16	25.98	$69.16*10^5$	6.840	Up-regulation
miR-15b	Lung cancer	16‘	26.18	$77.98*10^5$	6.892	Up-regulation
miR-15b	Lung cancer	17	25.38	$110.35*10^5$	7.043	Up-regulation
miR-15b	Lung cancer	17‘	25.52	$98.40*10^5$	6.993	Up-regulation

miR-15b	Lung cancer	18	25.12	$136.06*10^5$	7.134	Up-regulation
miR-15b	Lung cancer	18‘	25.36	$126.76*10^5$	7.103	Up-regulation
miR-15b	Lung cancer	19	24.87	$165.43*10^5$	7.219	Up-regulation
miR-15b	Lung cancer	19‘	24.66	$209.41*10^5$	7.321	Up-regulation
miR-15b	Lung cancer	20	26.47	$47.16*10^5$	6.674	Up-regulation
miR-15b	Lung cancer	20‘	27.20	$26.36*10^5$	6.421	Up-regulation
Cel_miR-39	Spike-in control	Std-1	29.52	$5.00*10^5$	5.699	N/A
Cel_miR-39	Spike-in control	Std-1	29.07	$5.00*10^5$	5.699	N/A
Cel_miR-39	Spike-in control	Std-2	32.40	$0.50*10^5$	4.699	N/A
Cel_miR-39	Spike-in control	Std-2	32.36	$0.50*10^5$	4.699	N/A
Cel_miR-39	Spike-in control	Std-3	35.64	$0.05*10^5$	3.699	N/A
Cel_miR-39	Spike-in control	Std-3	35.13	$0.05*10^5$	3.699	N/A
miR-21	NTC	1	N/A	N/A	N/A	N/A
miR-21	NTC	1‘	N/A	N/A	N/A	N/A
miR-15b	NTC	2	N/A	N/A	N/A	N/A
miR-15b	NTC	2‘	N/A	N/A	N/A	N/A

NTC= non-template control; N/A= not available

In the above table, the starting quantity of the miR-21 and miR-15b of the unknown samples (exposed to DME) was determined by using the Ct values obtained from the absolute quantification. Up-regulation of miR-21 and miR-15b was found in the majority of the samples. However, depending on the known concentrations of Cel_miR-39 spike-in control, up-regulation was found in 80% of all miR-21 samples, and 90% of DME exposed samples. While up-regulation was found in 85% of all miR-15b samples and 100% of DME exposed samples.

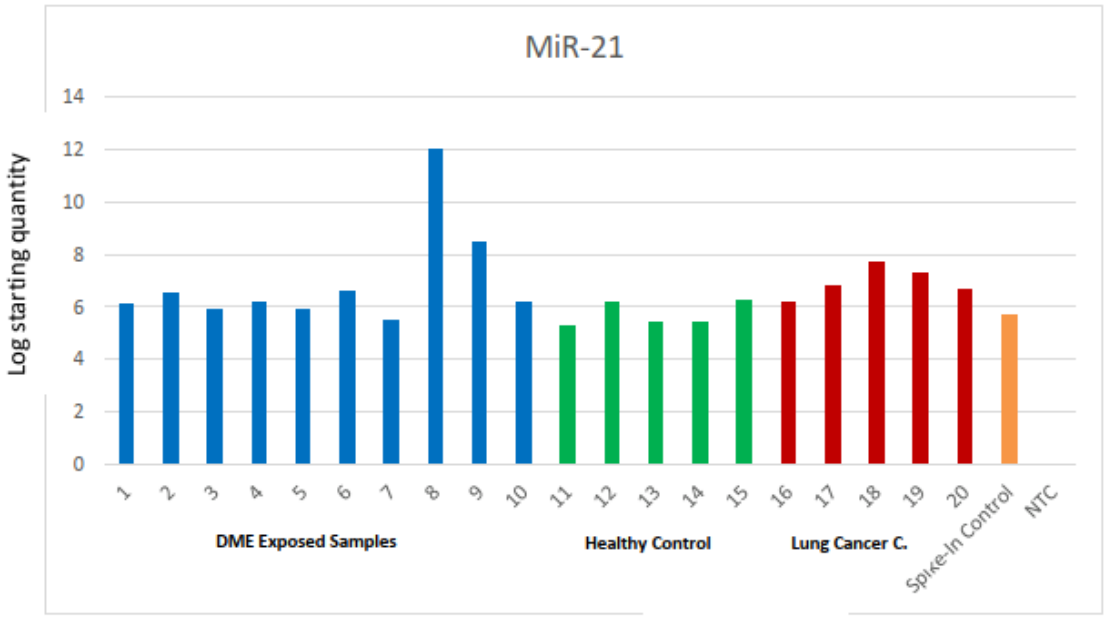


Figure 3.2 miR-21 expression in all samples including DME exposed samples (1 to 10), healthy control samples (11 to 15), lung cancer patients (+ve control) (16 to 20), spike-in controls, and non-template control (NTC) using qPCR.

The figure above shows the differences of expression of miR-21 in all samples. Logarithm of starting quantity is plotted in y-axis, while the sample number, spike-in control, and non-template control are plotted in the x-axis. The expression of the miR-21 in all DME exposed samples is expressed as increase or decrease relative to cel_miR-39 spike in control as a normalizer for comparison.

In the above figure, all DME exposed samples show over-expression of miR-21 in comparison to spike-in control except sample number 7. No amplification occur in NTC (no Ct value) as an evidence to absence of any contamination and primer dimer amplification.

All lung cancer patients samples (+ve control) (samples 16 to 20) show elevation in the expression of miR-21 compared with spike-in control.

While 80% of these samples show over-expression compared with healthy control samples. However, DME exposed samples number 8 and 9 are the most samples show over-expression of miR-21 as shown in the above figure. Healthy control samples number 11, 13, and 14 show miR-21 normal expression while samples 12 and 15 show little over-expression compared with spike-in control and lung cancer patients samples.

Depending on the above figure, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) results as follows:

Exposed	**Not-exposed**
True +ve 9	False +ve 2
False –ve 1	True –ve 3

Sensitivity = 9/10 x 100% = 90%

Specificity = 3/5 x 100% = 60%

PPV = 9/11 x 100% = 81.8%

NPV = 3/4 x 100% = 75%

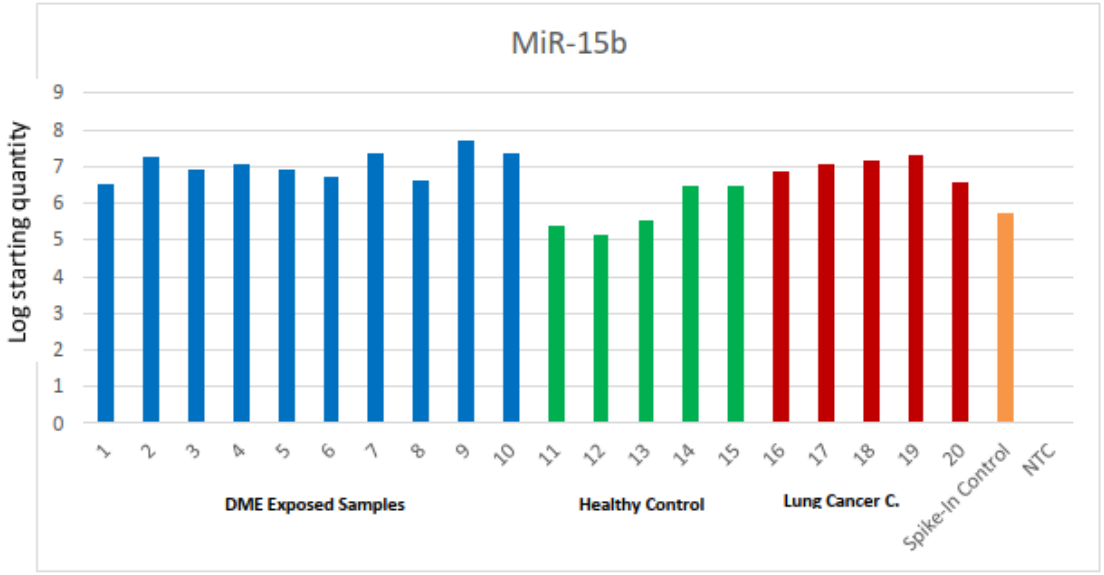


Figure 3.3 miR-15b expression in all samples including DME exposed samples (1 to 10), healthy control samples (11 to 15), lung cancer patients (16 to 20), spike-in controls, and non-template control (NTC) using qPCR.

The figure in the above shows the differences of expression of miR-15b in all samples. Logarithm of starting quantity is plotted in y-axis, while sample numbers, spike-in control, and non-template control are plotted in the x-axis. The expression of the miR-15b in all DME exposed samples is varying depending on their Ct values compared to Ct values of spike-in control which used in known concentrations.

All DME exposed samples and lung cancer patients show over-expression of miR-15b. While two out of five healthy samples show high expression of miR-15b. However, the expression of miR-15b in lung cancer patients and DME exposed samples are closed together. Since there is no amplification occur in NTC, there is no DNA contamination and primer dimer formation.

In the above figure, 100% of lung cancer patient's samples (+ve control) show over-expression in miR-15b compared with healthy control (cancer-free control) and spike-in control. Samples 7, 9, and 10 were the most DME exposed samples miR-15b expression compared with lung cancer and spike-in controls. Forty percent of healthy control show over-expression of miR-15b (samples 14 and 15) compared with spike-in control.

Depending on the above figure, sensitivity, specificity, PPV, and NPV results as follows:

Exposed	**Not-exposed**
True +ve 10	False +ve 2
False –ve 0	True –ve 3

Sensitivity = 10/10 x 100% = 100%

Specificity = 3/5 x 100% = 60%

PPV = 10/12 x 100% = 83.3%

NPV = 3/3 x 100% = 100%

Table 3.4 Sensitivity, specificity, PPV, and NPV for miR-21 and miR-15b expression in DME exposed samples and healthy controls.

MiRNA	**Sensitivity**	**Specificity**	**PPV**	**NPV**
MiR-21	90%	60%	81.8%	75%
MiR-15b	100%	60%	83.3%	100%

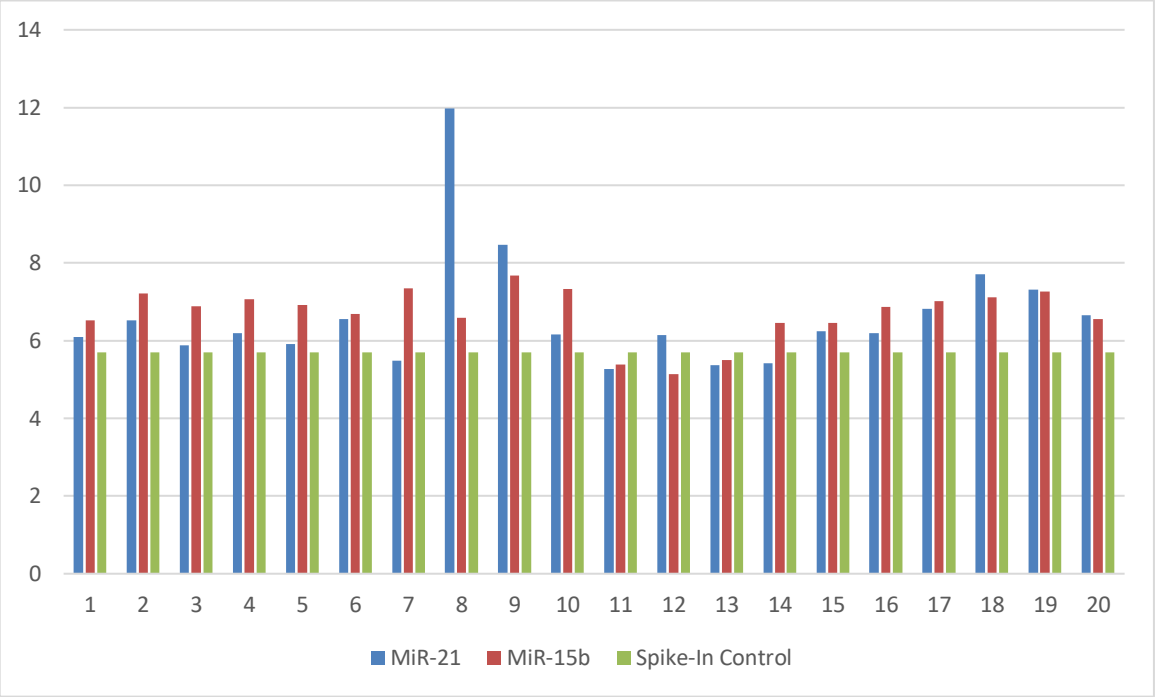


Figure 3.4 miR-21**,** miR-15b and spike-in control expression differences in all samples including DME exposed samples (1 to 10), healthy control samples (11 to 15), lung cancer patients (16 to 20).

The comparison between the expression of miR-21, miR-15b, and Cel_miR-39 spike-in control in all samples including DME exposed samples, healthy control, and lung cancer patients control were shown in the above figure. Nine samples out of 1 0 DME exposed samples including samples 1, 2, 3, 4, 5, 6, 8, 9, and 10 showing over-expression of miR-21 (90% of all DME exposed samples). While 100 % of DME exposed samples showing over-expression of miR-15b.

Two samples out of 5 healthy control samples including samples 12 and 15 showing over-expression of miR-21, while samples 14 and 15 showing over-expression of miR-15b (40% of all healthy control samples for both miR-21 and miR-15b). All lung cancer patients samples (+ve control)

showing up-regulation in both miRNAs compared with spike-in control as expected.

3.4 Statistical analysis

Table 3.5 Demographic and histopathologic data for plasma samples.

		Healthy C.	DME exposed	Cancer C.
		M (SD)	M (SD)	M (SD)
Log starting quantity of miR-21		5.68 (0.46)	6.92 (2.1)	6.93 (0.62)
Log starting quantity of miR-15b		5.78 (0.58)	7.01 (0.63)	6.96 (0.25)
Age		32.80 (13.08)	45.90 (12.06)	60.2 (4.62)
No. of years in transportation center		0	10.50 (2.87)	N/A
Exposure duration (hrs /day)		0	3.60 (0.681)	N/A
Total Exposure duration (hrs)		0	11918.40 (4411.88)	N/A
		n (%)	**n (%)**	**n (%)**
MiRNAs expression* (miR-21 and miR15b)	Normal regulation	6 (60%)	1 (5%)	0 (0%)
	Up-regulation	4 (40%)	19 (95%)	10 (100%)
Smoking history		0 (0%)	0 (0%)	4 (80%)**
Male		4 (80%)	10 (100%)	5 (100%)
Adenocarcinoma		N/A	N/A	4 (80%)
Squamous cell carcinoma		N/A	N/A	1 (20%)

M= Mean; SD= Standard deviation; N/A= not available; n = number

* For expression, considered 40 participants for both (miR-21 and miR-15b) since no differences found

** One of them Ex-Smoker 30 pack/years (quitted 2 years ago)

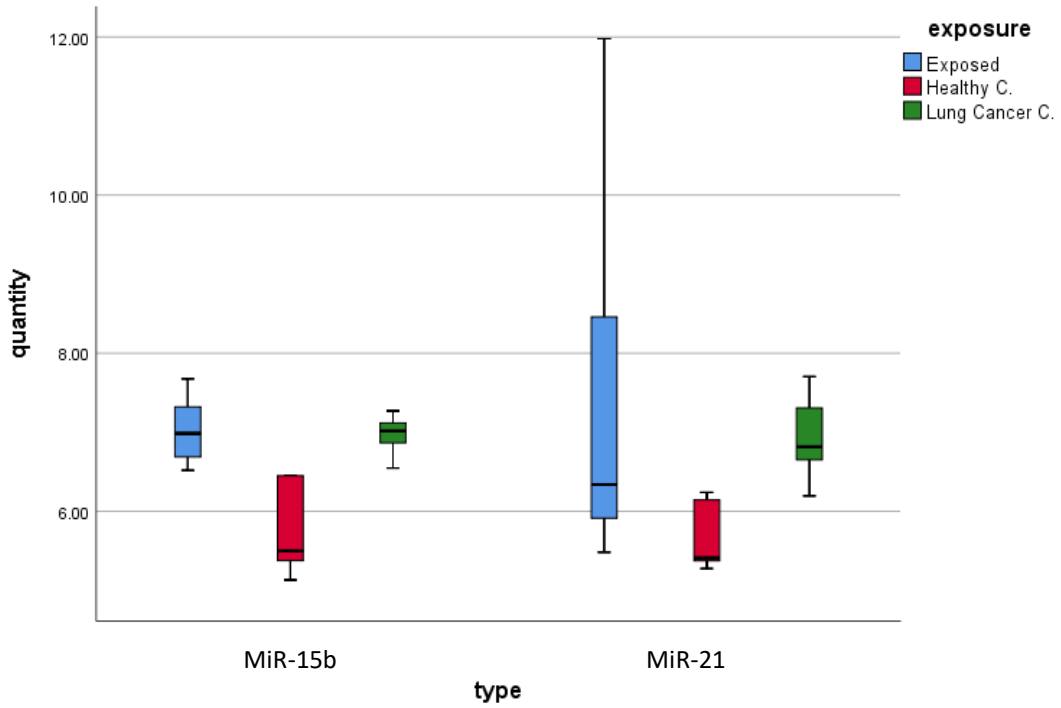


Figure 3.5 Box plot shows the correlation between plasma levels of miR-21 and miR-15b in healthy control (cancer-free control), DME exposed people, and lung cancer patients. The line inside the boxes denote the medians. The boxes mark the interval between 25th and 75th percentile. The Whiskers denote the interval between the 5th and 95th percentiles.

The above figure shows the comparison between all groups in the quantity of both plasma miRNAs (miR-21 and miR-15b). Healthy controls of miR-21 has the lower mean (5.68, SD=0.46), while the DME exposed people of miR-15b has higher mean (7.01, SD=0.63). Based on the significant difference in means between these three groups (ANOVA, $F=5.713$, $P<0.05$), healthy controls are responsible for the significant difference compared with DME exposed people and lung cancer patients based on post hoc comparison.

Table 3.6 The Correlation between the expression of miRNAs and the exposure to DME. Odds ratio, and confidence interval are shown.

		Sample			
		Healthy C.	**DME Exposed**	***P***	**Odds Ratio (CI)**
MiRNAs Expression	**Normal-regulation**	6 (60.0%)	1 (5.0%)	.002	28.5 (2.6-306)
	Up-regulation	4 (40.0%)	19 (95.0%)		

Table 3.7 Pearson correlation coefficient shows the correlation between DME exposure duration and quantity of both miRNAs (miR-21 and miR-15b).

		Quantity of miRNA
Total Exposure duration (hrs)	Pearson Correlation	0.570**
	Sig. (2-tailed)	0.001
	N	30

** Correlation is significant at the 0.01 level (2-tailed).

Note: There is no difference between miR-21 and miR-15b quantity and exposure duration.

Based on data showed in the above table, there is significant correlation between the quantity of plasma miRNA for both (miR-21 and miR-15b) and the exposure duration ($p<0.05$).

3.5 DNA check

Amplified cDNA using qPCR was checked by running the product on agarose gel after qPCR for making sure from the specificity of the qPCR assay (Figure 3.6).

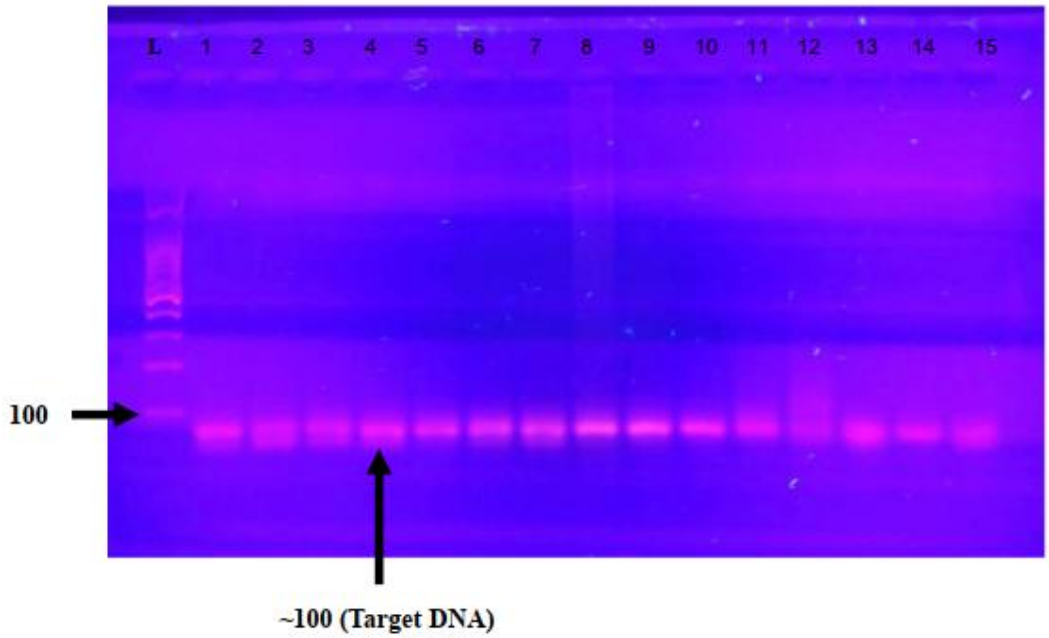


Figure 3.6 Representative samples for the amplified cDNA, (samples from 1 to 15).
L= 100bp DNA ladder

In the above figure, samples from 1 to 15 related to miR-21, samples 1 to 10 are DME exposed, samples 11 to 15 are for healthy control.

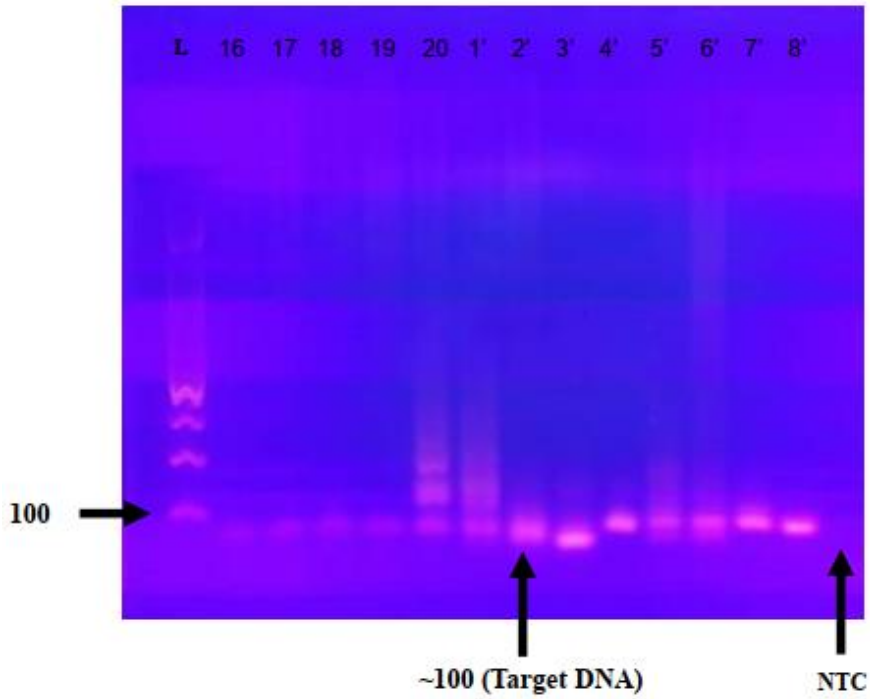


Figure 3.7 Representative samples for the amplified cDNA, (samples from 16 to 20, and 1' to 8').
L= 100bp DNA ladder
NTC= non-template control

In the above figure, samples from 16 to 20 related to miR-21 of lung cancer control, samples from 1’ to 8’ related to miR-15b (samples 1’ to 8’ are for DME exposed people).

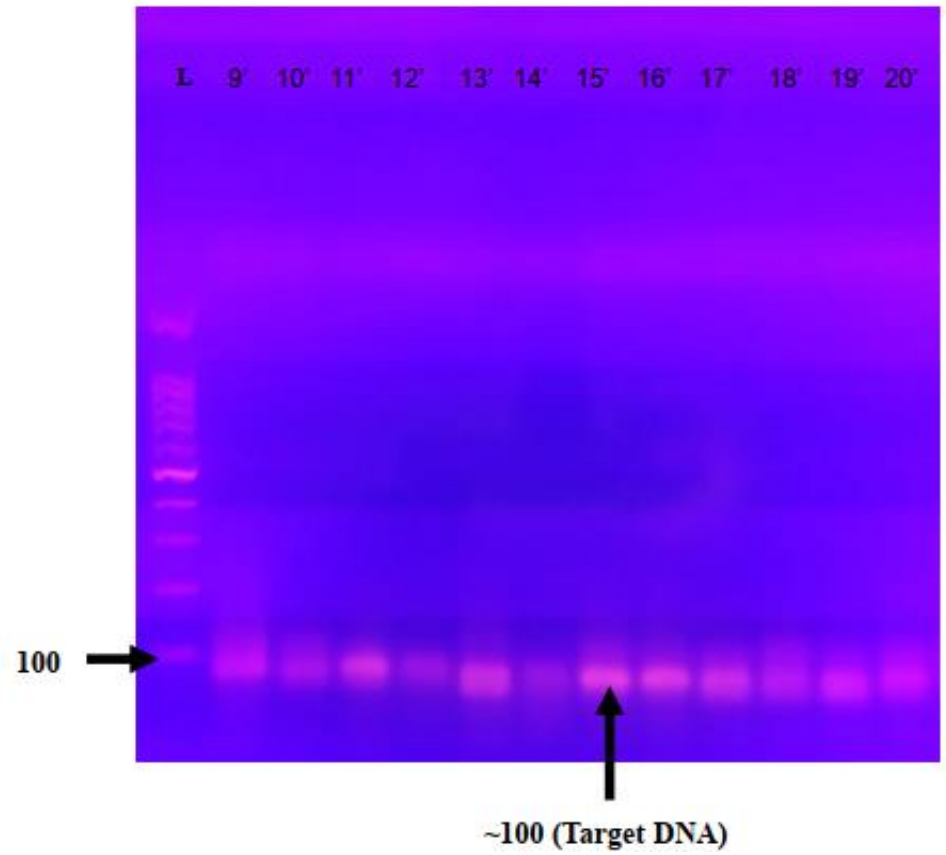


Figure 3.8 Representative samples for the amplified cDNA, (samples from 9’ to 20’ related to miR-15b).

The above figures show the separation of amplified cDNA for all samples using agarose gel electrophoreses (2% agarose gel, 90V, 90 minutes). Samples from 1 to 20 represent miR-21 amplified samples by real-time PCR. Samples from 1’ to 20’ represent miR-15b amplified samples using real-time PCR. L refers to 100bp DNA ladder. The molecular size (base pair) of the smaller band of DNA ladder are shown on the left side of the figures. Target cDNA (~100 bp) (22 nucleotides miRNA + 32 nucleotides adaptor primer + about 40 nucleotides poly (A) tail) found in all

wells in a sizes closed together. NTC refers to non-template control, proving the absence of primer dimer and DNA contamination.

3.6 Discussion

Efforts are focusing on the early detection of cancer including lung cancer to reduce the mortality. Human cancer exhibit an alteration in the miRNAs profile with tumor suppressive and oncogenic activity (99). MiRNAs were proved their ability in early detection of lung cancer and considered as a biomarker depending on many studies on this field. Many recent studies investigated the relationship between the upregulation of miR-21 and different human cancers (107, 123). It has been reported that miR-21 suppress PTEN which can induce apoptosis, control cell growth, and angiogenesis (124, 125). A study by Hennessey et al (100) proved the ability of miR-15b to distinguish NSCLC and healthy controls with high degree of sensitivity. The expression of miRNAs varies in plasma samples of high cancer risk people who exposed to different carcinogens including DME. In our study, we focused on the plasma levels of miR-21 and miR-15b in people who working at high intensity of DME which may related to lung tumorigenesis.

In real-time PCR, the starting quantity of DNA generated depends on Ct value. Ct value related to the cycle number at which the fluorescent signal crosses the threshold. So, if a high amount of the DNA template found at the start of the reaction, a few amplification cycles needed to give a fluorescent signal, and low Ct value obtained.

A standard curve was generated using 10-fold serial dilutions of known concentrations template determine the optimization of qPCR assay. In our results, depending on standard curve and Ct values generated by real-time PCR, the correlation coefficient (R^2) is 0.995, which represents how well the data fit the standard curve line. The value of R^2 for qPCR reaction should be greater than 0.980.

Ideally, the quantity of DNA template will double during each cycle in the exponential phase of the PCR amplification. This means that in each cycle, the copy number increases 2-fold which related to reaction efficiency of 2. Amplification efficiency, E, is measured from the slope of the standard curve. The optimal slope of the standard curve is -3.32.

The efficiency is calculated as follows:

$$E = 10^{-1/slop} \qquad \% \text{ Efficiency} = (E-1) \times 100\%$$

$$E = 10^{-1/-3.216} = 2.046$$

$$\% \text{ Efficiency} = (2.046 - 1) \times 100\% = 104.6\%$$

Depending on the results above, the reaction efficiency indicates that there is no nonspecific product amplification since the amplification efficiency should be 90 – 105%

In our results, 90% of DME exposed samples show over-expression of miR-21 and 100% for miR-15b. Forty percent of healthy controls show over-expression of both miR-21 and miR-15b. While 100% of lung cancer

patients samples control show over-expression of both miR-21 and miR-15b (Table 3.5). However, miR-21 and miR-15b are not specific biomarkers for lung cancer only, but also for different types of cancer including breast cancer, colon cancer, hepatocellular carcinoma, and glioblastoma (121, 122).

The expression of miR-21 in DME exposed samples 8 and 9 was the most with a starting quantity 7466.18 x 10^5 and 3763.4 x 10^5 copies/µl respectively. These results indicates the high exposure intensity of DME for these people working at transportation center which be about 6 hours daily for 15 years depending participants history and questioners.

Although all lung cancer patients participate in this research are under treatment at An-Najah National University Hospital, the results show significant elevation in the expression of both miR-21 and miR-15b compared with healthy and spike-in control with sensitivity equals 90% and 100% for both miR-21 and miR-15b respectively (Table 3.4). The relatively low false negative results for both miR-21 and miR-15b means the ability to identify most of lung cancer patients with a disease. The high sensitivity and relatively high specificity of both miR-21 and miR-15b indicate that they could discriminate plasma samples of DME exposed from healthy controls, and they will be a novel noninvasive biomarker for early detection of lung cancer. Recently, Shen et al (123) concluded that the plasma level of many miRNAs including miR-21 is useful for discriminate healthy controls from non-small cell lung cancer (NSCLC) patients with 86.22% sensitivity and

96.55% specificity, which supports the hypothesis that miRNAs may use as novel biomarkers for early detection of lung cancer.

On another hand, high NPV obtained from results using real-time PCR for quantification plasma miR-21 and miR-15b of high risk people with high intensity exposure to DME, indicates the ability to depend on this screening for early detection and there is less needs for other expensive and invasive tests such as chest biopsy and chest CT (Table 3.4).

In Our results, there is a statistically significance difference between three groups (healthy control, DME exposed, and lung cancer patients control) and the quantity of miRNAs in plasma samples. Importantly, both miR-21 and miR-15b were significantly over-expressed in DME exposed people and lung cancer patients compared with healthy control (Figure 3.5).

Depending on the miR-21 and miR-15b expression in plasma samples of healthy control, DME exposed samples. This study provides an evidence that occupational exposure to DME correlates with elevation of the expression of both miR-21 and miR-15b, which considered as biomarkers for high risk of lung cancer depends on the results that showed an odds ratio of OR= 28.5 (95% CI 2.6-306) for both miRNAs (Table 3.6).

The specificity of qPCR assay using agarose gel electrophoresis was checked. Expectedly, the size of the bands of DNA products is about 100 bp (Figure 3.6, 3.7 and 3.8). However, the poly (A) polymerase adding poly (A) tail for miRNA which be $\leq$ 150 base. The size of miR-21 and miR-15b is 22

nucleotides, and 25 nucleotides for adaptor primer. So, the expected size of the DNA product is about 100 bp (Figure 2.7 and 2.8).

Chapter Four

Conclusions and Recommendation

There is great needs to develop an early detection screening tests of lung cancer since the rate of mortality is increased when the disease diagnosed in its later stages. In conclusion, our study strengthen the arguments which report plasma miR-21 and miR-15b could be a potential non-invasive and cost effective biomarker for early detection and diagnosis of lung cancer.

Due to the few reports on the plasma biomarkers for early detection of different cancer types in Palestine, the results of this study have confirm the significance of using miRNAs in early detection of cancer. Many further studies on different miRNAs such as miR-155, miR-15a, miR-126, miR-127, and miR-197 which implicated in human lung cancer are needed.

We recommend adapt this screening research on a larger cohort on high risk people exposed to high intensity of DME in many different places among Palestinian population. Also, we recommend to follow up the DME exposed people and test the expression of miR-21 and miR-15b for another 2 years. This future researches will reinforce the clinical utility of miRNAs as a first line biomarker screening test for lung cancer.

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جامعة النجاح الوطنية

كلية الدراسات العليا

استخدام جزيئات MicroRNAs في بلازما الدم للكشف المبكر عن سرطان الرئة بين الأشخاص المعرضين لانبعاثات عوادم الديزل في فلسطين

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الملخص

الخلفية العلمية: يعتبر سرطان الرئة من أكثر أنواع السرطانات انتشارا في فلسطين، ويعد من أكثر الأمراض المسببة للوفاة بين الذكور، حيث يشكل نسبة تصل إلى 8% من مجمل حالات السرطان. يرتبط سرطان الرئة بشكل أساسي بعوامل بيئية مختلفة منها عوادم السيارات، حيث يؤدي ذلك إلى خلل على مستوى المادة الوراثية وتغيير في مستوى جزيئات microRNAs في بلازما الدم. تعتبر جزيئات miRNAs والتي تتكون من 19 – 25 نيوكليوتيد أحد أهم المؤشرات الحيوية المرتبطة بعدة أنواع من السرطانات منها سرطان الرئة. تقوم جزيئات miRNAs بعدة وظائف في مرحلة ما بعد نسخ شريط mRNA إما عن طريق تحطيم شريط mRNA أو منع عملية انتاج البروتين (الترجمة)، حيث تقوم بتنظيم قرابة 30% من جينات الإنسان التي لها دور في انقسام وتمايز الخلايا، والموت المبرمج لها.

الأهداف: الكشف المبكر عن سرطان الرئة لدى الأشخاص المعرضين لشدة مرتفعة من انبعاثات عوادم السيارات في مركز مدينة نابلس التجاري، بهدف التقليل من احتمالية الإصابة بسرطان الرئة وبالتالي تقليل نسبة الوفيات. بالإضافة إلى استخدام جزيئات miR–21 و miR–15b والتي لا تحتاج إلى جراحة وذات تكلفة مادية منخفضة كتقنية للكشف المبكر عن سرطان الرئة. كما قدمت الدراسة تقييما للتأكد من سلامة العمل داخل مجمع السيارات من ناحية صحية.

منهجية الدراسة: تم حساب كمية جزيئات كلا من miR–21 و miR–15b في بلازما الدم لعشرين عينة تشمل أشخاص معرضين لانبعاثات عوادم السيارات، أشخاص أصحاء (غير

معرضـين لعوادم السـيارات)، وأشـخاص مصـابين بسـرطان الرئة، من خلال اسـتخلاص جزيئات miRNAs في بلازما الدم، ثم إضـافة ذيل عديد الأدنين لها باسـتخدام انزيم بلمرة عديد الأدنين. تم صـناعة شـريط DNA المتمم (cDNA) باسـتخدام انزيم النسـخ العكسـي (RT)، وأخيرا تم تحديد كمية جزيئات miRNAs في الدم من خلال تقنية تفاعل البلمرة المتسلسل بالزمن اللحظي (real-time PCR).

النتائج: أثبتت الدراسـة أن ارتفاع مسـتوى جزيئات miR-21 كان عند 90% و miR-15b كان عند 100% من عدد الأشـخاص المعرضـين لانبعاثات عوادم السـيارات، بينما أظهرت ارتفاع النوعين عند جميع المرضـى المصـابين بسـرطان الرئة، و 40% عند الأشـخاص الأصـحاء مقارنة بجزيء miRNAs الضابط والمعروف تركيزه الطبيعي في الدم.

أظهرت أيضـا الاختبارات الاحصـائية أن نتيجة فحص الحساسية الاحصـائي كانت 100% بينما النوعية 60% . كذلك كانت القيمة التنبؤية الموجبة 81.8% لجزيئات miR-21 و 83.3% لجزيئات miR-15b، بينما كانت القيمة التنبؤية السـالبة 75% بالنسـبة لجزيئات miR-21 و 100% بالنسـبة لجزيئات miR-15b. كما وأظهرت النتائج أيضـا أن هناك علاقة قوية بين التعرض لعوادم السـيارات وارتفاع مسـتوى جزيئات miRNAs في بلازما الدم والتي تعتبر مؤشـر للإصابة بسرطان الرئة كما أثبتت العديد من الدراسات.

الاسـتنتاج: نسـتنتج أن جزيئات miR-21 و miR-15b يكمن اسـتخدامها كمؤشـر حيوي غير جراحي في الكشـف المبكر عن سرطان الرئة، وأن العمل داخل مجمع السيارات غير لائق صـحيا وقد يكون أحد أسباب الإصابة بسرطان الرئة لدى الأشخاص العاملين فيه.